

HYPOGLYCEMIC BRAIN INJURY

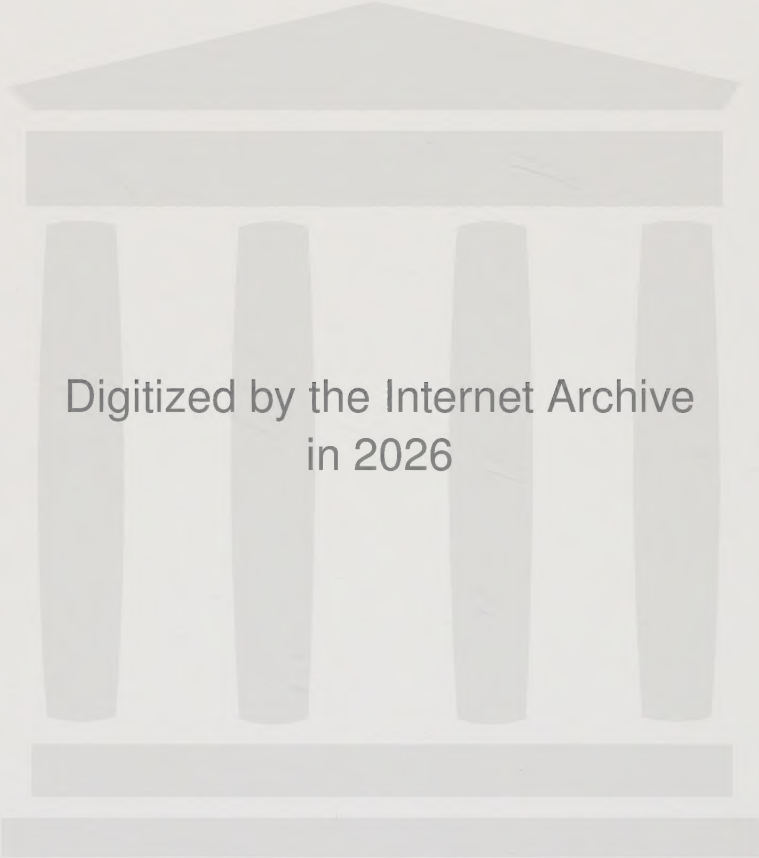
**An experimental study of the influence of profound
insulin-induced hypoglycemia on cerebral
metabolism and morphology in the rat.**

Carl-David Agardh



Lund 1981





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An experimental study of the influence of profound
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by

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Lund 1981



Printed in Sweden
Studentlitteratur
Lund 1981

The present thesis is based on the following papers:

- I. Agardh, C.-D., Folbergrová, J. and Siesjö, B.K. (1978) Cerebral metabolic changes in profound, insulin-induced hypoglycemia and in the recovery period following glucose administration. J. Neurochem. 31, 1135-1142.
- II. Agardh, C.-D., Chapman, A.G., Nilsson, B. and Siesjö, B.K. (1981) Endogenous substrates utilized by rat brain in severe insulin-induced hypoglycemia. J. Neurochem. 36, 490-500.
- III. Agardh, C.-D., Kalimo, H., Olsson, Y. and Siesjö, B.K. (1980) Hypoglycemic brain injury. I. Metabolic and light microscopic findings in rat cerebral cortex during profound insulin-induced hypoglycemia and in the recovery period following glucose administration. Acta Neuropathol. (Berl.) 50, 31-41.
- IV. Kalimo, H., Agardh, C.-D., Olsson, Y. and Siesjö, B.K. (1980) Hypoglycemic brain injury. II. Electronmicroscopic findings in rat cerebral cortical neurons during profound insulin-induced hypoglycemia and in the recovery period following glucose administration. Acta Neuropathol. (Berl.) 50, 43-52.
- V. Agardh, C.-D., Chapman, A.G., Pelligrino, D. and Siesjö, B.K. (1981) Influence of severe hypoglycemia on mitochondrial and plasmamembrane function in rat brain. J. Neurochem. (submitted)
- VI. Agardh, C.-D., Kalimo, H., Olsson, Y. and Siesjö, B.K. (1981) Hypoglycemic brain injury. Metabolic and structural findings in rat cerebellar cortex during profound insulin-induced hypoglycemia and in the recovery period following glucose administration. J. CBF Metab. (in press)
- VII. Agardh, C.-D. and Siesjö, B.K. (1981) Hypoglycemic brain injury. Labile metabolites reflecting cellular energy state, cyclic nucleotides and free fatty acids in the cerebellum of the rat after 30 and 60 min of severe insulin-induced hypoglycemia. J. CBF Metab. (accepted for publication)

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BACKGROUND

The brain has an absolute need for a continuous supply of oxygen and glucose for its oxidative metabolism. A reduction in either of these will cause cerebral dysfunction and, eventually irreversible nerve cell damage. Up to now, most interest has been focused on the clinical problems related to a reduced cerebral blood flow (ischemia), while less attention has been paid to the effect of a reduction in blood glucose concentration (hypoglycemia) on cerebral metabolism.

Hypoglycemia is associated with more than 100 diseases (Marks 1972). Its occurrence during treatment of diabetes mellitus and in the premature infant probably represents the two most common conditions. In patients with juvenile diabetes mellitus, insulin-induced hypoglycemia has been reported as the most frequent complication (Paz-Guevara et al. 1975) and the cause of death in 2.8-3.5 per cent (Paz-Guevara et al. 1975, Deckert et al. 1979).

Symptoms of hypoglycemia often occur when the blood glucose concentration falls below 2.5 mmol/l but they show individual variations and depend on the rate of onset and duration of hypoglycemia. Early symptoms (e.g. sweating, restlessness, hunger and tachycardia) which are often associated with an acute reduction in the blood glucose concentration, are probably caused by increased epinephrine secretion from the adrenals. With progressive and sustained hypoglycemia, numerous cerebral symptoms develop and often affect the cerebral cortical function first. Early symptoms of personality changes and irritability are followed by reduced levels of consciousness ending in stupor and coma. During the progressive decrease in blood glucose concentration, major seizures, focal neurological deficits and visual defects may occur (see Wilkinson and Prockop 1976).

Hypoglycemia as a cause of neuronal cell damage.

Prolonged hypoglycemic coma has been shown to cause brain damage in man (Lawrence et al. 1942, Meyer 1963, Banker 1967, Andersson et al. 1967, Brierley 1976, Kalimo and Olsson 1980), monkey (Brierley et al. 1971a,b, Myers and Kahn 1971), and rat

(Pogády and Skodáček 1979). The nature and time course of the neuronal alterations have been regarded as identical to the 'ischaemic cell changes' seen after hypoxia/ischemia (Meyer 1963, Brierley et al. 1971a, Brierley 1976). These changes have been considered to have a characteristic time course and a characteristic localization. The time course is assumed to involve a transition from 'microvacuolation' (presumed to reflect swelling of mitochondria), to 'ischemic cell change', eventually with 'incrustations', the end result being homogenizing cell change with dissolution of the neuron. The term selective vulnerability denotes the preferential localization of damaged neurons to certain regions. Thus, the cerebral cortex, especially the small pyramidal neurons in cortical layer 3 and to a lesser degree, neurons in cortical layer 5, the hippocampus and the striatum seem to be the most sensitive (Brierley 1976) while the cerebellum is less vulnerable (Meyer 1963). Concentration of injured neurons to the border zones between the arterial territories and Purkinje cell damage in the cerebellum common in hypoxia/ischemia are not seen in hypoglycemia (Myers and Kahn 1971) unless complicated by hypotension (Brierley et al. 1971b). Thus, the hypoglycemic insult can be aggravated by superimposed hypoxia and/or hypotension, a complication that is often seen in clinical practice during profound hypoglycemic coma.

Cerebral blood flow and oxygen and glucose metabolism during hypoglycemia.

A comatose state, whatever its cause, is considered to be associated with a decreased cerebral metabolic rate for oxygen (CMRO_2) (Sokoloff 1977). The introduction of insulin-induced hypoglycemia in the treatment of schizophrenia (Sakel 1937) has presented the opportunity to study the influence of hypoglycemia on cerebral circulation and metabolism in man. Thus, studies have shown cerebral blood flow (CBF) to be either unchanged (Kety et al. 1948, Eisenberg and Seltzer 1962, Gottstein and Held 1967) or increased (Della Porta et al. 1964) during hypoglycemia. Moderate hypoglycemia has been shown to be associated with normal (Gottstein and Held 1967) or even increased CMRO_2 (Eisenberg and Seltzer 1963). During coma,

Della Porta (1964) found normal $CMRO_2$ while in other studies coma was accompanied by a progressive decrease in $CMRO_2$ (Kety et al. 1948, Fazekas et al. 1951). Furthermore, hypoglycemia reduced the utilization of glucose to a greater extent than oxygen. These results have been borne out by animal experiments, where normal oxygen consumption has been found despite a substantial decrease in cerebral glucose consumption (CMR_{gl}) (Pappenheimer and Setchell 1973, Norberg and Siesjö 1976). Since under normal conditions an almost stoichiometric relationship exists between the cerebral oxygen and glucose uptake, the decrease in glucose utilization during hypoglycemia with a normal, or near normal oxygen consumption, indicates that other substrates than glucose provided by the blood are used for oxidation.

The influence of hypoglycemia on cerebral intermediary metabolism.

During the last 30 years, several experimental studies have been performed in attempts to evaluate the cerebral biochemical alterations that take place during hypoglycemia. In 1947, Olsen and Klein reported decreased levels in the brain of glucose, glycogen, phosphocreatine (PCr) and adenosine triphosphate (ATP) during insulin-induced hypoglycemia in artificially ventilated cats with an almost abolished EEG activity. Moreover, early work showed its influence on free amino acids in the brain, with a decrease in glutamate and a rise in aspartate concentrations (Dawson 1950, Cravioto et al. 1951, Samson et al. 1959).

Subsequent studies have confirmed these results and broadened our knowledge about the progressive biochemical changes occurring in the brain tissue during hypoglycemia. These can be divided into two stages which are mainly governed by the degree of hypoglycemia. In the first stage, in which blood glucose concentration often exceeds $1 \mu\text{mol/g}$, experimental animals show clinical signs of hypoglycemia (lethargy, seizures, loss of righting reflex and stupor) and the electroencephalogram (EEG) shows an altered pattern progressing from slow-waves and poly-spike activity to burst-suppression. During this period, extensive loss of most glycolytic and citric

acid cycle intermediates is seen in mice with unchanged levels of energy-rich phosphates (Goldberg et al. 1966, Ferrendelli and Chang 1973, Gorell et al. 1976, 1977). A close correlation between the blood glucose concentration and changes in cerebral cortical metabolites has been shown in artificially ventilated rats (Lewis et al. 1974a). In that study, a progressive decrease occurred in most glycolytic and citric acid metabolites when the arterial blood glucose concentration fell below $3\mu\text{mol/g}$. With a further decrease in blood glucose concentration below $2\mu\text{mol/g}$ there was an associated decrease in glutamate, glutamine, GABA and alanine concentrations with increased levels of aspartate and ammonia. The tissue glucose and glycogen were almost depleted when the arterial blood glucose fell below $1.7\mu\text{mol/g}$ and $1\mu\text{mol/g}$, respectively.

With progressive hypoglycemia, the second stage supervenes. The blood glucose concentration then falls below $1\mu\text{mol/g}$, the animals become comatose and the spontaneous EEG-activity ceases. This stage is biochemically characterized by gross energy failure with decreased tissue concentrations of ATP and PCr and a corresponding increase in adenosine diphosphate (ADP) adenosine monophosphate (AMP) and creatine (Cr); furthermore, the early changes in the free amino acid pool are aggravated (Tews et al. 1965, Hinzen and Müller 1971, Lewis et al. 1974a,b, Norberg and Siesjö 1976). Evidence exists that the cerebellum is less affected than the cerebral cortex (Hinzen and Müller 1971).

When the gross energy failure sets in, and this seems to be an abrupt phenomenon, potassium is released to the extracellular space at about the same time as the EEG becomes isoelectric (Astrup and Norberg 1976).

While there is extensive knowledge about the influence of hypoglycemia on the metabolism of carbohydrate and energy-rich phosphates, less information is available about its effect on the brain lipids. Since the brain is one of the organs of the body that is richest in lipids, and since brain lipids have important structural and functional properties, a loss of phospholipids during hypoglycemia might cause serious functional disturbances and underly the development of irreversible cell

injury. During hypoglycemia there is evidence of decreased syntheses (Dawson and Richter 1950, Ansell and Dohmen 1957, Ansell and Spanner 1959) and increased breakdown of phospholipids (DeRopp and Snedeker 1961, Knauff and Böck 1961) resulting in reduced levels of phospholipid phosphorus or individual phospholipid concentrations (Randall 1940, McGhee et al. 1951, Stone et al. 1972). In one study, the decrease in the phospholipid concentration was correlated to the blood glucose concentration and did not occur until the blood glucose fell below 1.3mmol/l and the cerebral cortex and the cerebellum were affected to about the same extent (Hinzen et al. 1970).

Substrate utilization during hypoglycemia

Under normal conditions, the oxygen consumption of the brain equals its production of carbon dioxide. This leads to a respiratory quotient of unity indicating that the substrate utilized is of carbohydrate origin. Furthermore, the molar ratio between the cerebral uptake of oxygen and glucose from the blood is about 6, which means that the carbohydrate substrate leading to a respiratory quotient of unity is glucose taken up from the circulation.

During hypoglycemia, several studies have shown that the oxygen/glucose ratio increases to above 6. This can be explained by the fact that substrates other than glucose are derived from the blood or that endogenous substrates are oxidized. Several substances in the blood are potential substrates for cerebral metabolism (for review, see Sokoloff 1977). However, the entrance to the brain is regulated by the special properties of the cerebral vasculature, known as the blood-brain barrier (BBB) (for a recent review, see Rapoport 1980). The BBB has poor permeability for ions, proteins and water-soluble non-electrolytes. The transport of certain substances is facilitated by a carrier-mediated transport system which is stereospecific, follows Michaelis-Mentens kinetics and needs no energy. So far, such carriers have been identified for hexoses, short-chain monocarboxylic acids, amino acids, nucleic acid precursors, and choline. Since the transport is driven by the concentration gradient of the substance across the membrane, certain substances that reach low concen-

trations during insulin-induced hypoglycemia can probably be ruled out. Thus, ketone-bodies which can serve as substrates for cerebral oxidative metabolism in fasted humans (Owen et al. 1967), and, in proportion to their arterial concentrations, in rats (Hawkins et al. 1971) are less likely candidates. Furthermore, measurements have failed to show any arterio-venous differences in lactate in experimental animals during hypoglycemia (Nemoto et al. 1974), or in free fatty acids in starved man (Owen et al. 1967). It seems less probable, therefore, that substances derived from the blood could account for the cerebral oxidative metabolism during profound hypoglycemia.

The nature of the endogenous substrates is only partly known. Energy stores of glycogen are small and amount to about 2-3 $\mu\text{mol/g}$ in the rat cerebral cortex. Other available substrates are provided by glycolytic metabolites and citric acid cycle intermediates. Furthermore, some free amino acids (glutamate, glutamine, aspartate and GABA) exist in high concentrations in cerebral tissues and can be used as substrates through transamination reactions and by oxidative deamination of glutamate. In one study (Norberg and Siesjö 1976), however, the amino acid pool remained constant after the first 5 min of hypoglycemia with an isoelectric EEG and the authors concluded that the upheld CMR_{O_2} must have occurred at the expense of non-carbohydrate, non-amino acid substrates. Since hypoglycemia is associated with a breakdown of lipids (e.g. Hinzen et al. 1970) oxidation of fatty acids could provide this 'missing' substrate. This has gained some support from experiments with glucose-free perfusion of isolated cat brain with maintained electrical activity and normal oxygen consumption (Abood and Geiger 1955). Since there was a breakdown of proteins and lipids and there was a respiratory quotient as low as 0.5, it was suggested that these may have served as substrates. The brain is available to oxidize fatty acids in vitro (Vignais et al. 1958, Beattie and Basford 1965) and in vivo (Allweis et al. 1966). However the rate of oxidation seems to be slow compared with that of glucose oxidation, and at present, there is no clear evidence that fatty acids can replace glucose as a substrate in situations of glucose deprivation.

Hypoglycemia as a cause of brain dysfunction.

The signs of cerebral dysfunction which occur during hypoglycemia without gross cerebral energy failure and with an unchanged CMR_{O_2} are not readily understood, but have sometimes been attributed to 'transmission failure' due to an imbalance between amino acids that are supposed to have excitatory and inhibitory actions as transmitters in the brain. Thus, a decrease in GABA and an increase in aspartate concentrations might underlie some of the neurological symptoms seen during hypoglycemia (Siesjö 1978).

Furthermore, during hypoglycemia a progressive increase occurs in ammonia concentration within the brain (Tews et al. 1965, Lewis et al. 1974a, Feise et al. 1976, Norberg and Siesjö 1976) with a rapid decrease to normal values after glucose administration (Tews et al. 1965). Since increased concentrations of ammonia are known to cause depression of cerebral function and epileptic activity (Tews et al. 1963, Hindfelt and Siesjö 1971) accumulation of ammonia during hypoglycemia might contribute to the symptoms.

It has also been suggested that brain edema, defined as an increase in brain volume due to increased water content, causes brain dysfunction during hypoglycemia, but the results differ. Arief (1974) reported a 12 per cent increase in brain water content during hypoglycemia in artificially ventilated rabbits, while other workers found a less pronounced increase or unchanged water content (Ellison 1958, Feise et al. 1976, Thurston et al. 1976). In neither of these, though, was it possible to study regional changes because of the technique used. It still has to be decided whether these or related alterations are responsible for the symptoms or are mere epiphenomena.

AIM OF THE PRESENT STUDY

This background formed the basis for the present experiments which were performed in order to study the time course and nature of morphological alterations in two brain struct-

ures (cerebral and cerebellar cortex), which in previous studies have been considered as 'vulnerable' and 'less vulnerable' respectively. In an attempt to reveal the underlying mechanisms behind the development of irreversible cell damage, the corresponding metabolic alterations were studied as well.

The experiments were performed on artificially ventilated rats in order to rule out the influence of hypotension and hypoxia, which, superimposed on hypoglycemia have been shown to aggravate the hypoglycemic insult (e.g. Brierley et al. 1971b, Ferrendelli and Chang 1973).

METHODS

Animals, induction of hypoglycemia and operative techniques.

Male Wistar Rats (260-440g) of a S.P.F. Wistar strain were used in all experiments. The animals were fasted over night before the experiments but had access to water. One hour before operation hypoglycemia was induced by an intraperitoneal injection of insulin (Insulin Novo Actrapid^(R), in a dose of 40 I.U. $\cdot$ kg⁻¹. The insulin was dissolved in 0.75 ml Krebs-Hensleit solution before injection. Control animals were given 0.75 ml of this solution.

Anaesthesia was induced with 2-3 per cent halothane; the animals were then tracheotomized, immobilized with i.v. injection of tubocurarine chloride (0.5 mg $\cdot$ kg⁻¹) and ventilated with a Starling type respirator, which was adjusted to deliver 70 per cent N₂O and 30 per cent O₂. In animals used for isolation of mitochondria, halothane was not used, but analgesia was induced with 80 per cent N₂O and 20 per cent O₂. Surgical regions were locally infiltrated with lidocaine (10 mg $\cdot$ ml⁻¹), 0.4-0.5 ml per animal. The animals were then paralyzed with 1.5 mg tubocurarine chloride i.p. and, following tracheotomy, ventilated as described above.

Body temperature was kept around 37°C. One femoral artery was cannulated for continuous blood pressure recording using an electromanometer and for sampling of blood, and one femoral vein was cannulated for injections. The electroencephalogram (EEG) was continuously recorded in all animals from gold-plated copper-bolts inserted into the skull bone in the

fronto-parietal region. In animals used for sampling of cerebral tissue for biochemistry, a skin incision was made over the skull bone to accommodate a plastic funnel for later freezing of the tissue in situ. In animals used for measurement of cerebral blood flow, a burr hole was made over the distal part of the superior sagittal sinus for sampling of cerebral (cortical) venous blood. For measurements of arterio-venous differences for glucose, oxygen and ketone bodies, one retroglenoidal vein was cannulated for sampling of cerebral venous blood as previously described (Nilsson and Siesjö 1976, 1980). After the operative procedures had been completed, the animals were given heparin i.v., halothane was discarded and the animals were continuously ventilated on 70 per cent N₂O and 30 per cent O₂ to yield an arterial P_{CO}₂ of 30-40 mm Hg and a P_O₂ around 100 mm Hg. Repeated arterial samples were anaerobically taken from the catheter in glass capillaries for determination of pH, P_{CO}₂ and P_O₂, or collected directly in liquid nitrogen for later analyses of glucose and ketone bodies.

Sampling of tissue

a. Biochemistry. At the end of the experiments the tissue was frozen in situ with liquid nitrogen (Pontén et al. 1973). Samples of the frozen cerebral tissue were chiselled out and stored at -80°C.

b. Morphology. At the termination of the experiments, a quick thoracotomy was performed, and a cannula inserted in the ascending aorta via the left ventricle. Following a short rinse with saline, the brain was fixed by perfusion with glutaraldehyde. In some of the experiments, the animals were similarly perfused with FAM-fixative. After perfusion, the brain was allowed to stabilize in situ for about 1 h after which it was removed and stored in the same fixative until processed for microscopy.

c. Mitochondria. At the end of the experimental period the animal was decapitated and the brain, apart from the cerebellum and medulla oblongata, immediately removed for isolation of mitochondria. Brain mitochondria were isolated by a modification of the technique described by Clark and Nicklas (1970). The details have recently been described from the

laboratory (Rehncrona et al. 1979).

Analytical techniques

a. Blood gases and pH. The PCO_2 , PO_2 and pH in arterial blood were measured immediately after sampling using microelectrodes operated at 37°C with appropriate temperature corrections.

b. Carbohydrate substrates, labile phosphates, amino acids and ammonia. Cerebral cortical tissue from the frontoparietal regions and cerebellar cortical tissue were extracted as described previously (Folbergrová et al. 1972a). Metabolites were determined with the fluorometric techniques of Lowry and Passonneau (1972). Analytical conditions for measurements of blood glucose concentrations and tissue concentrations of phosphocreatine, creatine, ATP, ADP, AMP, glycogen, glucose, pyruvate, lactate, α -ketoglutarate, glutamate, glutamine, aspartate, alanine, GABA and ammonia have been previously described (Folbergrová et al. 1972a,b, 1974a,b).

c. Lipids. The lipids were extracted by chloroform-methanol (Folch et al. 1957) and separated by thin layer chromatography. Phospholipids were eluted from the plates as described by Åkesson et al. (1970). Phospholipids were quantified by phosphorus determination according to the method of Belfrage et al. (1970). The concentrations of individual phospholipids were calculated from the total lipid phosphorus and the percentage distribution of phosphorus between the different phospholipid classes. Total fatty acids were transesterified and free fatty acids were esterified as described by Åkesson et al. (1970). The fatty acid methyl esters were separated by gas liquid chromatography.

d. Ribonucleic acids. The total cortical ribonucleic acid (RNA) was extracted and analysed with a modified Schmidt-Thannhauser procedure (Schmidt and Thannhauser, 1945, Munro and Flech, 1966). The concentration of RNA was determined by measuring the ultraviolet absorbance at 260 nm and 280 nm. The results were checked by also determining the phosphorus content in the extract (Itaya and Ui, 1966, Belfrage et al. 1970). For calculation of RNA content, an average extinction coefficient at 260 nm = 11.0 mM^{-1} was used according to the base composi-

sition of brain (cortical) RNA reported by Mahler et al. (1966).

e. Cyclic nucleotides. Cyclic AMP was measured by a protein-binding technique using Cyclic AMP Assay kit, Amersham, England. Cyclic GMP was measured using the radioimmunoassay technique described by Steiner et al. (1972) using commercial kit (Cyclic GMP (^{125}I -labelled) RIA kit, New England Nuclear, Boston, U.S.A.

f. Blood concentrations of β -hydroxybutyrate (β -HB) and acetoacetate (AcAc). Blood concentrations of β -HB and AcAc were measured with a fluorometric modification of the spectrophotometric techniques of Mellanby and Williamson (1974) and Williamson and Mellanby (1974).

Measurements of cerebral blood flow (CBF) and cerebral metabolic rate for oxygen (CMR_{O_2}).

Cerebral blood flow was measured by a modification (Norberg and Siesjö 1976, Berntman et al. 1979) of the Kety and Schmidt technique (1948) using (^{133}Xe) as the tracer. The arterial and venous (superior sagittal sinus) differences in total oxygen content (TO_2) (AVD_{O_2}) was measured by a polarographic technique (Borgström et al. 1974) in repeated arterial and venous samples. The CMR_{O_2} was calculated by multiplying the CBF with the mean of the AVD_{O_2} .

Measurements of arterio-venous differences for glucose (AVD_{gl}) and oxygen (AVD_{O_2}).

For calculation of $\text{AVD}_{\text{O}_2}/\text{AVD}_{\text{gl}}$ ratios paired samples of arterial and cerebral venous blood were repeatedly taken. The cerebral venous blood was sampled from one retroglenoid vein cannulated according to the technique described by Nilsson and Siesjö (1980). The total oxygen content was immediately determined as described above. Samples for blood glucose were collected directly in liquid nitrogen for later analyses as described above.

Measurement of extracellular potassium.

Extracellular potassium concentration ($[\text{K}^+]_{\text{e}}$) was measured with a double-barrelled K^+ microelectrode, which was inserted into the cortical tissue through a small slit in the dura. The construction and use of the electrode has been previously described (Astrup and Norberg 1976).

Measurement of mitochondrial respiratory activity.

Oxygen uptake was measured polarographically in a closed and magnetically stirred chamber with an oxygen electrode operating at 24°C. The incubation medium consisted of 0.225 M mannitol, 0.075 M sucrose, 1 mM EGTA, 0.01 M H₃PO₄ and 0.01 M HCl neutralized to pH 7.4 (= MSTP_i), MSTP_i + 2.5 per cent bovine serum albumin (BSA), MSTP_i + 100 mM KCl and MSTP_i + 2.5 per cent BSA + 100 mM KCl, respectively. Malate plus glutamate was used as NAD-linked substrate and succinate as FAD-linked substrate, respectively. Mitochondrial protein concentration was measured according to Lowry et al. (1951) and cytochrome (a+a₃) content was determined with a dual wave length spectrophotometer (Aminco D-W 2).

Calculations and statistics.

The 'energy state' of the tissue was calculated in terms of the adenylate energy charge (E.C.) according to Atkinson (1968). Statistical differences were evaluated with Student's t-test when the material appeared to follow a normal distribution. Otherwise, the Mann-Whitney U-test was applied. Correlations were calculated by the conventional method for linear correlation.

RESULTS AND COMMENTS

As stated in the Background, the blood pressure was continuously recorded and blood gas analyses repeatedly performed in the present experiments. Thus, the influence of hypotension, hypoxia and hypercapnia was excluded. Late in the hypoglycemia and in the recovery period, there was a slight fall in arterial pH.

The duration of severe hypoglycemia was defined as the period with an abolished EEG activity ('isoelectric EEG'). This occurred when the arterial blood glucose concentration fell to around or below 1 µmol/g (cf. Lewis et al. 1974b). In the control animals, there was a gradual rise in blood glucose concentration, most likely explained by the stressful situation during the experimental procedures. During recovery, the blood glucose concentration was normal or increased to above normal.

Cerebral blood flow and oxygen consumption.

Hypoglycemia with an isoelectric EEG for 15 and 30 min resulted in an increase in cerebral cortical blood flow to about 270 and 230 per cent, respectively, of the control value. The CMR_{O_2} was unchanged during the first 15 min of hypoglycemia with an isoelectric EEG, but dropped to about 70 per cent of the control value after 30 min. Since the CBF has been shown to be pressure passive during profound hypoglycemia (Nilsson et al. 1981), the reduction in the CBF after 30 min compared with the 15 min group is partly explained by the lower blood pressure in the former group.

Cerebral arterio-venous differences for glucose and oxygen.

In order to calculate the non-glucose substrate requirement during hypoglycemia, paired blood samples were taken from one retroglenoidal vein, (which essentially drains the whole brain) and from one femoral artery. During the progressive lowering of the blood glucose concentration, the AVD_{gl} gradually decreased from 0.42 ± 0.04 $\mu\text{mol/g}$ (mean $\pm$ SEM), when the arterial blood glucose concentration was 2.00 ± 0.09 , to 0.16 ± 0.03 $\mu\text{mol/g}$, when it had fallen to 0.56 ± 0.10 after 30 min of isoelectric EEG. The extraction of glucose from the arterial blood increased from the approximately 10 per cent reported during normal conditions (Crone 1965, Norberg and Siesjö 1976) to 20 - 30 per cent during hypoglycemia. In spite of this, there was a decrease to about 0.5 in the molar ratio between cerebral glucose utilization to oxygen consumption ($\text{glucose}/O_2$), indicating that considerable amounts of non-glucose substrates were oxidized. The arterial concentrations of ketone bodies decreased during hypoglycemia, and arterio-venous differences for lactate and pyruvate were undetectable (unpublished data), making them less probable as substrates (see Background). In summary, the present results show that hypoglycemia with an isoelectric EEG is associated with a 2-3-fold increase in CBF, a normal CMR_{O_2} during the first 15 min of hypoglycemia and a reduction in CMR_{O_2} to about 70 per cent of control when the hypoglycemic period is extended to 30 min. During these periods, the oxygen consumption exceeds the glucose utilization, and since there were no indications that

substrates were derived from the blood, the oxidative metabolism must have been upheld from endogenous stores.

Cerebral cortical metabolic changes during hypoglycemia.

a. Glycolytic intermediates and α -ketoglutarate (α -KG).

The cerebral cortical stores of glucose and glycogen were almost depleted after 5 min of hypoglycemia, and there was an extensive reduction in the concentrations of lactate, pyruvate and α -KG, but a prolongation of the hypoglycemic period revealed no further decrease.

During recovery, following glucose administration, cerebral cortical glucose concentration returned to normal, while the rate of glycogen synthesis was slow (about $0.01 \mu\text{mol/g/min}$). Both lactate and pyruvate concentrations increased to above normal. Since there was a greater increase in lactate concentration, the lactate/pyruvate ratio increased, although there was a tendency towards normalization with longer periods of recovery.

b. Energy-rich phosphates. In the present experiments, (Fig.1), the ATP concentration was reduced by about 60 per cent after 5 min of hypoglycemia. Previous results have

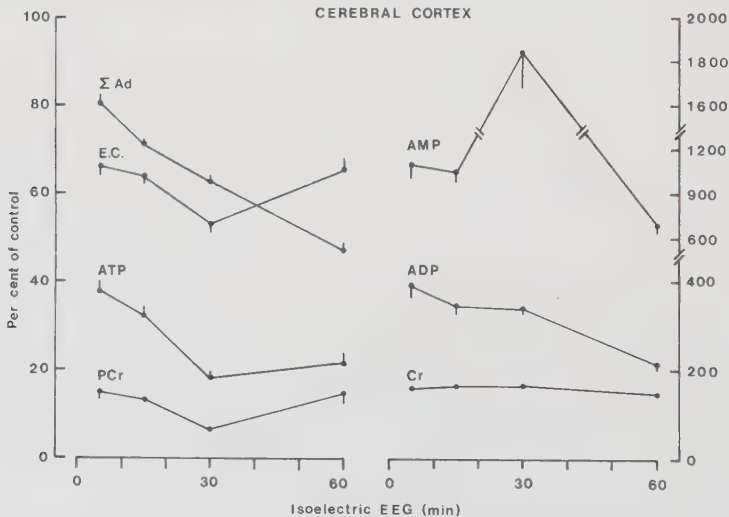


FIG.1. Cerebral cortical concentrations of labile energy rich phosphates, sum of adenine nucleotides (ΣAd) and the calculated energy charge (E.C.) of the tissue during hypoglycemia with an isoelectric EEG for 5 to 60 min. The values are given in per cent of control and represent mean $\pm$ S.E.M.

shown that gross energy failure and reduction in ATP concentration appears when the blood glucose concentration falls to or below 1 $\mu\text{mol/g}$ and the EEG becomes isoelectric (Lewis et al. 1974b, Norberg and Siesjö 1976). In the period 5 to 30 min there was a further progressive fall and the ATP concentration reached 18 per cent of the control level after 30 min. No further reduction occurred when the hypoglycemia was extended to 60 min. There was an associated increase in the concentrations of ADP and AMP. However, these concentrations reached their highest values during the 5 to 30 min period, and then gradually decreased. As a result, the adenylate pool decreased to 47 per cent of control after 60 min of hypoglycemia. There were similar changes in PCr and creatine concentrations as well, but both PCr and creatine reached their extreme values after 5 min with no further decrease thereafter, and the sum of PCr and creatine did not fall. In the recovery period (Fig.2), the ATP concentration increased during the first 30 min after glucose administration,

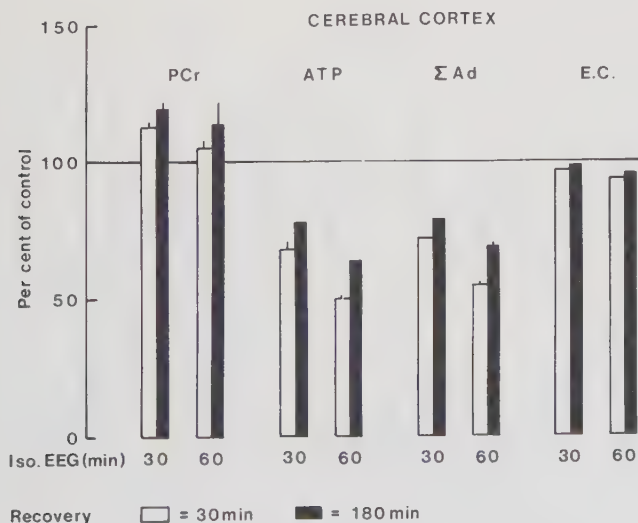


FIG.2. Cerebral cortical concentrations of PCr, ATP, sum of adenine nucleotides (ΣAd) and the calculated energy charge (E.C.) of the tissue after 30 and 180 min of recovery following hypoglycemia with an isoelectric EEG for 30 and 60 min, respectively. The values are given in per cent of control and represent mean $\pm$ S.E.M.

with only a small further increase in the period 30 to 180 min. As a result, the adenine nucleotide pool remained reduced by 20 and 30 per cent after 180 min of recovery following hypoglycemic periods of 30 and 60 min, respectively. The ADP and AMP concentrations were close to normal. PCr was restored to supernormal concentrations with reciprocal changes in creatine concentration.

c. Amino acids and ammonia. The present results show that hypoglycemia is associated with a severe derangement of the free amino acids in the brain tissue. As shown in Fig.3, there were progressive decreases in the concentrations of GABA, alanine, glutamate and glutamine during the first 30 min of hypoglycemia with no further reduction when the hypoglycemic period was prolonged to 60 min. The concentration of aspartate increased to about 400 per cent during the first 30 min, but decreased substantially during the 30 and 60 min period. Thus, the sum of aspartate and glutamate concentrations decreased during this period. The ammonia concentration was markedly increased during all periods.

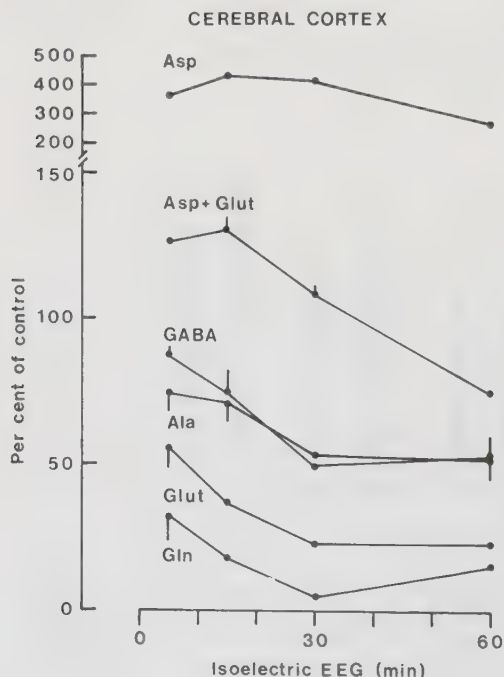


FIG.3. Cerebral cortical concentrations of aspartate, GABA, alanine, glutamate and glutamine during hypoglycemia with an isoelectric EEG for 5 to 60 min. The values are given in per cent of control and represent mean $\pm$ S.E.M.

After glucose administration (Fig.4), GABA concentration returned to normal values, even after 60 min of hypoglycemia. There was a progressive rise in glutamine concentration with normal concentration after 180 min of recovery following 30 min of hypoglycemia, but a lingering reduction by 40 per cent after 60 min of hypoglycemia. The glutamate concentration increased to 80-90 per cent of control values. Aspartate decreased below normal while alanine increased above normal. As a result, the free amino acid pool remained reduced by 2.6 $\mu\text{mol/g}$ and 5.4 $\mu\text{mol/g}$ after 180 min of recovery following 30 and 60 min of hypoglycemia, respectively. The ammonia concentration decreased to subnormal values.

CEREBRAL CORTEX

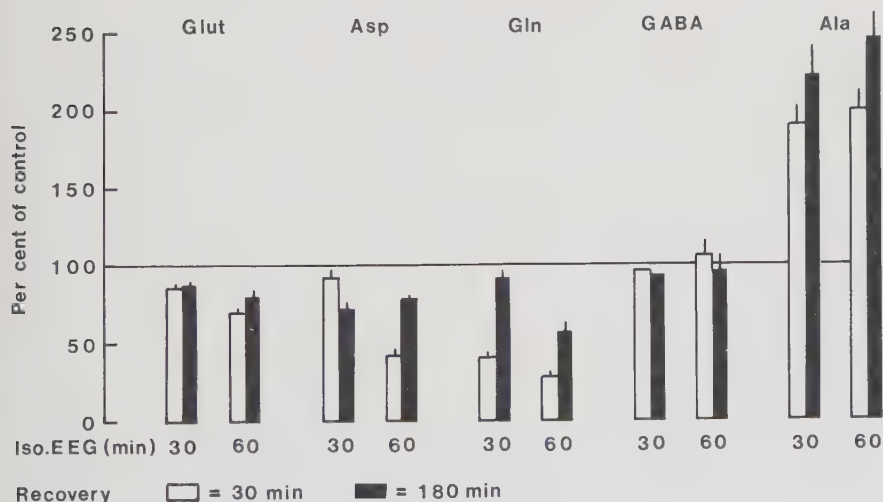


FIG.4. Cerebral cortical concentrations of glutamate, aspartate, glutamine, GABA and alanine after 30 and 180 min of recovery following 30 and 60 min of hypoglycemia with an isoelectric EEG, respectively. The values are given in per cent of control and represent mean $\pm$ S.E.M.

In summary, profound hypoglycemia was associated with an almost complete depletion of glycolytic metabolites that were already present after the first 5 min of hypoglycemia with an isoelectric EEG. Previous results have shown that this occurs

before the development of gross energy failure and cessation of EEG activity (see Background). There was a progressive decrease in the concentration of ATP during the first 30 min of hypoglycemia, with no further reduction thereafter. However, the adenylate pool continued to decrease, most probably as a result of catabolism of AMP to adenosine and inosine monophosphate (IMP), of which both increase during hypoglycemia (Chapman et al. 1981). The fall in pyruvate concentration may lead to decreased levels of acetyl CoA with reduced formation of citrate in the citric acid cycle, and to accumulation of oxalacetate (OAA) previously reported by Goldberg et al. (1966) and Lewis et al. (1974a). As a result, the aspartate aminotransferase (E.C. 2.6.1.1.) reaction will shift to the right, leading to the formation of aspartate and α -KG from glutamate and OAA. Since the increase in aspartate concentration exceeds the decrease in glutamate during the first 30 min of hypoglycemia, further glutamate might be available from glutamine, and to a lesser extent, from GABA and alanine. However, in the period 30 and 60 min, there was a significant fall in aspartate concentration. This could be explained by metabolism of aspartate in the purine nucleotide cycle, but since these reactions require guanosine triphosphate (GTP), which is substantially decreased during hypoglycemia (Chapman et al. 1981), such a metabolism is less likely. It seems more probable that oxidative deamination of glutamate and the decrease in the amino acid pool by about 10 μ mol/g during the period 15 to 60 min will shift the aspartate aminotransferase reaction towards glutamate formation.

The accumulation of ammonia during hypoglycemia is explained by decreased synthesis and increased breakdown of glutamine, oxidation of glutamate by the glutamate dehydrogenase (E.C. 1.4.1.2) reaction and by catabolism of purine nucleotides, all of which lead to the accumulation of ammonia. The present data do not reveal if there is any breakdown of proteins during hypoglycemia.

In the recovery period, there was a tendency towards restoration of most metabolites. However, there was a lingering reduction in the adenine nucleotide pool. Resynthesis of purine nucleotides may occur by de novo synthesis, by kinase re-

actions with nucleosides as substrates, or by salvage pathways where purine bases react with phosphoribosylpyrophosphate. Since de novo synthesis proceeds at a very slow rate (Gerlach et al. 1971), this route seems less probable. More likely, IMP and adenosine, which accumulate during hypoglycemia and normalize in concentration in the recovery period (Chapman et al. 1981), are converted to AMP and GMP after glucose administration. The increase in the PCr/creatine ratio could reflect changes in intracellular pH and/or Mg^{2+} concentration which both influence the creatine kinase (E.C. 2.7.3.2) reaction (Kuby and Noltmann 1962).

d. Lipids. During hypoglycemia, the total phospholipid-bound phosphorus in the cerebral cortex decreased by 8 per cent and the total fatty acid content by 10 per cent. These changes were present after hypoglycemia with an isoelectric EEG for 5 min and there was no further decrease when the hypoglycemic period was prolonged to 60 min. There were similar decreases in choline phosphoglycerides (CPG) and ethanolamine phosphoglycerides (EPG) while inositol plus serine phosphoglycerides (IPG+SPG) did not change significantly. Among the individual fatty acids, there was a small decrease in palmitic acid (16:0) and in stearic acid (18:0), and a slightly more pronounced decrease in oleic acid (18:1) and arachidonic acid (20:4) which were reduced by 16 and 9 per cent, respectively. Under normal conditions the levels of free fatty acids (FFA) in the brain are low (Bazán et al. 1971). During hypoglycemia a sixfold increase in the FFA concentration was present already after 5 min of hypoglycemia with sustained levels when the hypoglycemic period was prolonged to 60 min.

In the recovery period, the changes in the phospholipids and total fatty acids persisted. However the FFA level was normal after 90 min of recovery.

Since the brain tissue is rich in phospholipids, which account for about 70 per cent of the lipid content of human gray matter (Suzuki 1976), and degradation of phospholipids may occur during pathological conditions, this may have detrimental effects on membrane structure and function.

In summary, hypoglycemia causes a breakdown of phospholipids, mainly CPG and EPG, and a decrease in the total fatty

acid pool without a preferential loss of any special class of fatty acid. There was a sixfold increase in the FFA level. After glucose administration, the concentration of the FFAs normalized, while there was no evidence for resynthesis of the phospholipids in the recovery period studied.

Cerebellar cortical metabolic changes during hypoglycemia.

The metabolic changes in the cerebellar cortex were qualitatively of the same character as those in the cerebral cortex. However, some differences were seen that are worth pointing out. The breakdown of ATP was less pronounced (Fig.5) and overt energy failure seemed to appear first after longer periods of hypoglycemia. Thus, the adenylate pool decreased to 66 per cent in the cerebellar cortex compared with 47 per cent in the cerebral cortex after 60 min of hypoglycemia with an isoelectric EEG. Furthermore, there was a considerable variation between animals concerning the cerebellar cortex. In some animals, the cerebellar cortex was only moderately influenced when gross energy failure developed in the cerebral cortex, while in other animals there was an extensive breakdown of energy-rich phosphates.

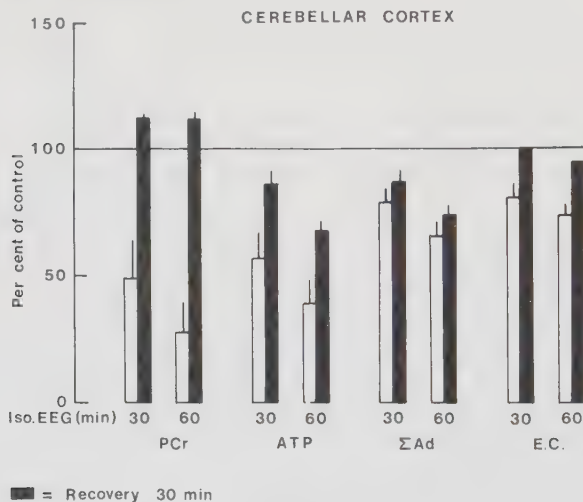


FIG.5. Cerebellar cortical concentrations of labile energy-rich phosphates, sum of adenine nucleotides (Σ Ad) and calculated energy charge (E.C.) of the tissue during hypoglycemia with an isoelectric EEG for 30 and 60 min as well as after 30 min of recovery. The values are given in per cent of control and represent mean $\pm$ S.E.M.

After 30 min of recovery, the adenine nucleotide pool was reduced to 87 and 74 per cent after 30 and 60 min of hypoglycemia, respectively. In both groups, lactate and pyruvate concentrations were increased, and the lactate/pyruvate ratio elevated. After 30 min of recovery following 30 min of hypoglycemia, (Fig.6), aspartate, glutamate and GABA concentrations were back to normal, while there was a reduction in the glutamine concentration by 20 per cent and an increase of alanine above normal by 50 per cent. After the same recovery period following 60 min of hypoglycemia, only GABA concentration returned to normal, while glutamate, glutamine and aspartate were reduced. As a result, the amino acid pool decreased by about 5 $\mu\text{mol/g}$ or by 22 per cent. In contrast to the cerebral cortex, there were no clear changes of the total phospholipid phosphorus concentration and the total fatty acid content. The FFA concentration increased, but not to the same extent as in the cerebral cortex since the increase was only significant after 60 min of hypoglycemia. As in the cerebral cortex, the largest relative increase occurred in the concentration of arachidonic acid. The increase in FFA concentration was clearly related to the adenylate energy charge of the tissue.

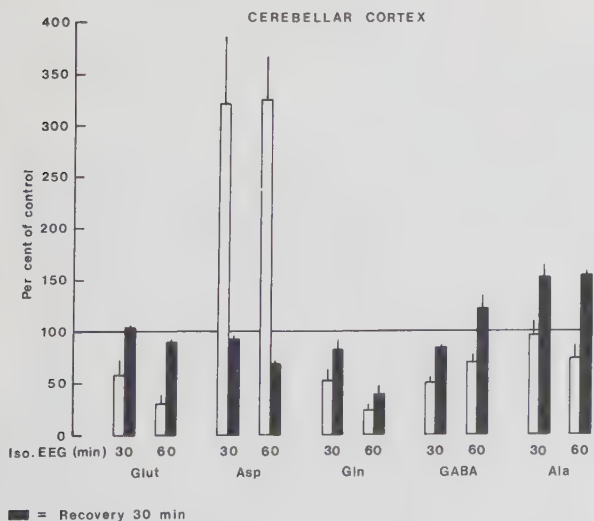


FIG.6. Cerebellar cortical concentrations of glutamate, aspartate, glutamine, GABA and alanine during hypoglycemia with an isoelectric EEG for 30 and 60 min as well as after 30 min of recovery. The values are given in per cent of control and represent mean $\pm$ S.E.M.

In summary, the cerebellar cortex showed the same qualitative changes in glycolytic intermediates, energy-rich phosphates and free amino acids as in the cerebral cortex. However, there was a greater variability between different animals and the alterations were less extensive and developed after longer periods of hypoglycemia. Furthermore, no clear changes occurred in the phospholipid concentration, and the increase in the FFA concentration was less pronounced.

The influence of hypoglycemia on cyclic nucleotides.

The concentration of cyclic 3', 5'-adenosine monophosphate (cAMP) increased in the cerebral cortex during hypoglycemia. However, with more profound hypoglycemia and a decrease in the adenylate energy charge (E.C.) below 0.8, there was a secondary decrease in cAMP concentration. Thus, the cAMP reached control values when the E.C. fell below 0.6. The concentration of cyclic 3',5'-guanosine monophosphate (cGMP) on the other hand, was increased after 5 min of hypoglycemia and the accumulation persisted even after the hypoglycemic period was prolonged to 60 min.

In the cerebellar cortex, hypoglycemia brought about a considerably higher increase in the concentration of cAMP and there was no correlation to the energy state of the tissue. The accumulation of cGMP, too, rose to higher values than in the cerebral cortex. Furthermore, there was a close correlation between the energy state, with cGMP reaching higher concentrations when the energy failure progressed.

In summary, the cAMP increased about 1.5 and 3.5-5 times in the cerebral and cerebellar cortex, respectively. In the cerebral cortex, there was a secondary decline when the hypoglycemia was more profound. The cGMP concentration increased about 2-2.5 times in the cerebral cortex and 5-7 times in the cerebellar cortex. However, the control level in the cerebellum was about 13 times higher than in the cerebral cortex and the increase was closely related to the energy state of the tissue.

Morphological changes during hypoglycemia

In the present studies, the neuronal alterations in cerebral and cerebellar cortex have been studied in artificially ventilated rats after profound hypoglycemia with an abolished EEG activity for either 30 or 60 min as well as during recovery and the influence of hypoxia and hypotension could be ruled out. In the clinical situation, however, it is often impossible to rule out this influence which is known to aggravate the hypoglycemic insult (see Background).

For reliable evaluation of morphological alterations in the brain it is essential to be able to rule out the ever-deceptive artefacts (see Cammermayer 1978), a task that is always difficult. Artefactual dark neurons are easily produced by improper or delayed fixation (Cammermayer 1972). In the present experiments, the tissue was fixed by perfusion of glutaraldehyde (GA) through an intra-aortic cannula after a brief rinse with saline. The brains appeared macroscopically solid and yellowish brown in colour, and microscopically the capillaries were empty with no or very few blood cells in their lumina, indicating proper fixation of the tissue. A certain type of dark neurons appeared during hypoglycemia but such neurons were not seen in the control animals processed identically for microscopy. Thus, this alteration in the hypoglycemic animals indicates a nerve cell injury which either is present in vivo at the moment of fixation, or, at least, reflects an altered reactivity of an injured cell towards the processing for microscopy. Furthermore the number of these dark neurons increased substantially when the hypoglycemic period was prolonged from 30 to 60 min, and decreased in animals with recovery, which corroborates the view that the alteration represents an in vivo injury.

a. Neuronal alterations in cerebral cortex. Two microscopically different types of nerve cell injury were seen during hypoglycemia, with no obvious transition between them. (In the following text called type I and type II). The distribution of the injured neurons in the cerebral cortex was uneven. Smaller neurons, especially in cortical layer 3,



FIG.7. Rat cerebral cortex after hypoglycemia with an isoelectric EEG for 60 min. Type I injury (arrowhead) and type II injury (arrow). J-B4 and toluidine blue. x 150.

seemed to be more vulnerable. The greatest number of affected neurons was located over the lateral aspects of the cortex, while the cortex at the vertex and on the medial surface was less affected. In areas of most severe involvement a sponginess of the neuropil was seen, reflecting tissue edema, and swelling of astrocytic processes. In the light microscope (LM) the type I injured neurons appeared darkly stained, triangular in shape with marked shrinkage and condensation of both their cytoplasm and nuclei. The plasma membrane was scalloped and commonly surrounded by small perineuronal vacuoles, which always seemed to be extracellular. The processes of these condensed neurons were often seen as dark bands traversing the neuropil. In the electron microscope (EM) the perineuronal vacuoles around the neurons were recognized as swollen astrocytic processes. The neuronal cytoplasm was darkly stained with compact ribosomes tightly packed in clusters around rough-surfaced endoplasmatic reticulum (RER). The mitochondria were often elongated without an increase in the electron lucency of their inner matrix and there were no signs of dilatation. The Golgi cisternae were often dilated. The nuclei were condensed and their inner membranes irregular.

In LM, the type II injured neurons appeared slightly swollen and in the JB-4 and paraffin embedded sections they had characteristic subplasmalemmal empty looking vacuoles which sometimes extended into the dendrites. However, in Epon-sections only subplasmalemmal clear zones but no vacuoles were found after 30 min of hypoglycemia and even after 60 min actual vacuoles were very rarely present. The nucleus appeared normal with finely dispersed chromatin and prominent nucleoli. In contrast to the type I injured neurons, no perineuronal vacuoles were seen. At the EM-level, the subplasmalemmal clear zones or vacuoles (depending on the histotechnique used) were due to the organelles mostly gathering around the nucleus so that the periphery of the cytoplasm was cleared. There seemed to be a decrease in the number of RER-cisternae as well as disintegration of both RER-bound and free ribosomes, no longer oriented in rosettes. The mitochondria were markedly contracted and elongated in shape. The nuclei still remained normal in shape with evenly distributed chromatin.

Prolongation of the hypoglycemic period from 30 to 60 min caused, qualitatively, essentially similar alterations, but, as expected, they were more accentuated and the proportions of injured cells increased substantially. During the recovery period, the number of neurons with type I injury decreased, but they had a similar distribution and appearance as during hypoglycemia, except for the somewhat increased electron lucency (but not extensive swelling) of the inner matrix of the mitochondria (not seen during hypoglycemia). Furthermore, after 60 min of hypoglycemia the type I injury in some neurons appeared to become even more severe during the recovery. The swollen perineuronal astrocytic processes seemed much less prominent and no longer were there any signs of tissue edema. Nor were any definite type II injured neurons seen during the recovery periods.

b. Neuronal alterations in cerebellar cortex. Compared with the extensive neuronal alterations seen in the cerebral cortex, the structural alterations in the cerebellar cortex were surprisingly few and discrete.

In LM and EM, virtually no alterations could be observed after 30 min. Even after 60 min of hypoglycemia the Purkinje and granule cells appeared unchanged except that in EM their mitochondrial matrix was slightly condensed, and, in some Purkinje cells, the number of RER cisternae appeared to be decreased. The only nerve cells that showed constant changes were small neurons, mostly basket cells, deep in the molecular layer. In LM, these cells appeared slightly swollen with pale cytoplasm in which clear vacuoles were seen. In EM, their mitochondria were distended with disruption of the cristae. The Golgi cisternae were dilated and membrane whorles were often seen in the cytoplasm. In addition, a few small angulated darkly stained cells similar to the type I injured neurons in the cerebral cortex were seen in the molecular layer. No clear alterations of the Fañanas astrocytes or perivascular astrocytic processes were seen. In the recovery period, no striking changes occurred, and even the swollen mitochondria in the basket cells persisted.

In summary, during hypoglycemia two different types of

injured neurons were seen in the cerebral cortex. Their appearance and localisation were definitely different from those seen during ischemia. Thus, no swollen mitochondria or dilated endoplasmatic reticulum were seen during hypoglycemia and the injured neurons were not localised to the arterial border zones as in ischemia.

The changes in cerebellar cortex were discrete and the only definite ones were localised to the basket cells deep in the molecular layer, which showed distended mitochondria with disrupted cristae and dilatation of the Golgi cisternae.

The influence of hypoglycemia on mitochondrial function and phospholipid content.

In the present experiments, the resting (state 4) and ADP-stimulated (state 3) oxygen consumption was measured in mitochondria, isolated after hypoglycemic periods with an isoelectric EEG for 30 and 60 min, as well as after a 90 min recovery period, and the respiratory control ratio (RCR) and ADP/O ratio were calculated. Glutamate plus malate and succinate were used as substrates and the effect of BSA and KCl on mitochondrial respiration was evaluated. In another series of experiments, the mitochondrial phospholipid content was measured after 30 min of hypoglycemia.

The results show that 30 min of hypoglycemia leads to an inhibition of state 3 respiration by 25-40 per cent when glutamate plus malate served as substrates, while the respiration was unchanged when succinate was the substrate. State 4 respiration did not change. As a result of the reduced state 3 respiration the RCR was reduced. However, the ADP/O ratio remained unchanged. Addition of BSA plus KCl partially reversed the inhibition of state 3 respiration, but could not prevent it altogether. During the recovery period, the inhibition of state 3 respiration was reversed.

After 60 min of hypoglycemia, the state 3 respiration with glutamate plus malate was further inhibited. When succinate served as substrate, there was a slight inhibition of state 3 respiration as well as a tendency towards a decline in the ADP/O-ratios. During recovery, the state 3 respiration became normal, while there was a rise in state 4 respiration

and a suggested decline in ADP/O-ratios, whatever substrate used.

Hypoglycemia with an isoelectric EEG for 30 min did not reveal any alterations in mitochondrial content of total fatty acids, major fatty acid classes, phospholipid phosphorus content or individual phospholipid classes.

Previous results have shown that hypoglycemia leads to energy failure in spite of a normal CMR_{O_2} (see Background) indicating uncoupling of oxidative phosphorylation. Other studies have shown that hypoglycemia is accompanied by a degradation of phospholipids (e.g. Hinzen et al. 1970) and accumulation of FFA (Agardh et al. 1980) in the cerebral cortex. Free fatty acids are known to disturb mitochondrial function by their inhibition of adenine nucleotide translocation across the inner mitochondrial membrane (Wojtczak 1976, Shrago 1978), inhibition of mitochondrial respiration (Pressman and Lardy 1956, Björntorp et al. 1964, Lochner et al. 1978) and uncoupling of oxidative phosphorylation (Pressman and Lardy 1956, Lehninger and Remmert 1959, Björntorp et al. 1964, Chefurka 1966, Vázquez-Colón et al. 1966, Wojtczak 1976).

In summary, hypoglycemia of 30 min duration leads to changes in mitochondrial function that are potentially reversible, but signs of mitochondrial failure develop in the recovery periods following 60 min of profound hypoglycemia. There were no signs of breakdown of mitochondrial phospholipids.

The influence of hypoglycemia on ribonucleic acid (RNA) content in cerebral cortex.

Prolonged hypoglycemia leads to a decrease in the number of RER-cisternae as well as a disintegration of both RER-bound and free ribosomes (see above). Since about 80 per cent of the RNA exists in the form of ribosomal RNA (Rappoport et al. 1966), the cerebral cortical content of RNA was calculated to show whether the EM findings were correlated to any change in RNA content.

The results reveal that total RNA content in rat cerebral cortex, following 30 min of hypoglycemia with an isoelectric EEG, decreased by about 10 per cent, corroborating the changes seen in EM.

The influence of hypoglycemia on plasma membrane function.

As described above, there is a lingering reduction in the ATP concentration in the cerebral cortex by about 20 and 35 per cent after 180 min recovery following hypoglycemia of 30 and 60 min duration, respectively, and a corresponding decrease in the adenine nucleotide pool by 20 and 30 per cent. Other (unpublished) results have shown the failure of electrophysiological recovery following 60 min of hypoglycemia with a better preserved function following 30 min of hypoglycemia. To evaluate if the differences between the hypoglycemic periods of 30 and 60 min duration in electrophysiological and metabolic recovery could be explained by changes in the integrity and function of the plasma membrane, the extracellular potassium concentration, $[K^+]_e$, was recorded in the recovery periods following 30 and 60 min of hypoglycemia.

The results show that $[K^+]_e$ became normal in the recovery period following 30 min of hypoglycemia. However, following 60 min of hypoglycemia, the $[K^+]_e$ progressively increased, reaching values of 8.0 ± 1.0 (Mean $\pm$ SEM) to 17.3 ± 1.0 mMol/l after 90 and 180 min of recovery, respectively.

DISCUSSION

In the present experiments the nature and time course of morphological and metabolic alterations in the brain during profound hypoglycemia with an isoelectric EEG for 5 to 60 min have been described. Two cerebral structures (cerebral and cerebellar cortex), previously considered as 'vulnerable' and 'less vulnerable', respectively, have been studied. In the following discussion, a comparison between the morphological changes and their eventual metabolic correlates will be made in an attempt to reveal the mechanisms underlying the development of irreversible cell damage during profound hypoglycemia. However, one should bear in mind the hazards involved when comparing the alterations seen in single cells and their subcellular elements and the metabolic changes in the tissue as a whole. The mere presence of morphologically injured cells cannot lead to the conclusion that all metabolic actions have ceased and conversely that metabolic functions may have stopped

before histologic changes appear.

Morphological alterations.

After both 30 and 60 min of hypoglycemia with an abolished EEG, two microscopically different types of nerve cell injury were seen in the cerebral cortex. The injured cells were predominantly localised to the lateral aspects of the cortex and to cortical layer 3. The type I injury is characterized by angulated, darkly stained neurons surrounded by swollen astrocytic processes, without swelling of the mitochondria. This type of cell change persisted in the recovery period suggesting irreversible cell damage. The type II injured neurons were larger ones, appearing slightly swollen with contraction of mitochondria, disintegration of ribosomes and loss of RER. These alterations were no longer seen after glucose administration, indicating reversible changes.

In the cerebellar cortex, however, the histopathological alterations seen were discrete. The only permanent changes appeared in the basket cells, showing clearly distended mitochondria and disruption of cristae and the Golgi cisternae were dilated. Also in contrast to the cerebral cortex, there was no swelling of the perivascular astrocytic processes. Hypoglycemic cell changes have previously been considered identical to those seen after hypoxia/ischemia (Brierley 1976). The dark neurons (type I injury) in the present studies resemble those seen after ischemia (Brown and Brierley 1973, Arsenio-Nunes et al. 1973, Garcia et al. 1973, 1977), but no microvacuolation was seen, and the mitochondria were not swollen. Moreover, neurons in the arterial border zones were not selectively vulnerable during hypoglycemia, further indicating a different pathogenesis from that in hypoxia/ischemia with a reduced perfusion pressure.

Conformational changes in mitochondria are governed by their metabolic state. Condensation of isolated mitochondria has been described in the presence of oxygen but without substrate available (Hackenbrock 1966, Hackenbrock et al. 1971). The condensed conformation is also seen when the rate of respiration is maximal (Lehninger 1976). In the present experiments, the CMR_{O_2} was normal despite a gross reduction in the

ATP concentration which could be explained by the uncoupling of the oxidative phosphorylation leading to a condensed mitochondrial state. The lack of mitochondrial swelling in the cerebral cortex during hypoglycemia in contrast to the swelling seen during ischemia may also be explained by differences in metabolic state in the two situations, e.g. lack of tissue acidosis (Pelligrino and Siesjö 1981) and a more oxidized state during hypoglycemia (Lewis et al. 1974a).

Changes in the energy state.

It has been natural to assume that cell damage during hypoglycemia is caused by or related to the energy failure. The present morphologic studies clearly show that extensive neuronal cell damage occurs in the cerebral cortex, while, in the same animal, the cerebellar cortex is much less affected. The question must therefore be raised if the energy state is better preserved in the cerebellar cortex and if this can explain the differences in vulnerability between the two regions. The present results have demonstrated that the metabolic deterioration in the cerebellar cortex has a slower onset, is less extensive and shows greater variability compared with the cerebral cortex. Since the endogenous stores of metabolites are not essentially different in the two structures, a more efficient transport of glucose to the cerebellum could explain the less extensive energy failure (Abdul-Rahman and Siesjö 1980). However, after 60 min of hypoglycemia, the metabolic changes in the cerebellum were comparable to those seen in the cerebral cortex after 30 min of hypoglycemia, at a time when about 20 per cent of the neurons in the neocortex showed alterations. These results raise the question whether a simple relationship exists between energy failure and the development of cell damage.

Changes in phospholipids.

In the cerebral cortex, there was a reduction in the phospholipid content and their related fatty acids by about 10 per cent, while no clear alterations were seen in the cerebellar cortex. Since degradation of membrane phospholipids must have serious consequences for the cell structure and membrane function, these differences may, at least in part, ex-

plain the differences between the morphological alterations in the two structures. Changes in brain lipids may occur by peroxidative degradation (Barber and Bernheim 1967, Rehnrcrona et al. 1980) or by phospholipase action (Sun et al. 1979). Peroxidative changes preferentially affect EPG, rich in polyunsaturated fatty acids. Since no such preferential loss of EPG or polyunsaturated fatty acids was seen during hypoglycemia, there is no evidence that free radical reactions initiating lipid peroxidation can underlie the development of tissue damage during hypoglycemia. Furthermore, accumulation of peroxide products (malondialdehyde) did not occur in the cerebral cortex after 30 min of hypoglycemia (Agardh, unpublished results).

Changes in free fatty acids.

Release of free fatty acids by phospholipase activity is known to occur in brain tissue (see Bazán 1976, Sun et al. 1979). The mechanisms behind the activation of phospholipases could tentatively be explained by Ca^{+2} entering the cells (or being released from intracellular stores) when energy failure develops and the ATP-concentration decreases (Markelonis and Garbus 1975). During hypoglycemia there is an accumulation of FFA both in the cerebral cortex and cerebellum. However, in the latter structure, the increase was less extensive and did not occur until after 60 min of hypoglycemia. In both structures, the relatively largest increase occurred in the concentration of arachidonic acid, which could serve as substrate for increased prostaglandin synthesis. Increase in the tissue concentrations of FFA and their acyl CoA esters has been shown to interact with many aspects of cell function. Thus, non-esterified fatty acids cause cellular edema in cortical slices (Chan and Fishman 1978), inhibition of adenine nucleotide translocase across the inner mitochondrial membrane (for review, see Wojtczak 1976), inhibition of mitochondrial respiration and uncoupling of oxidative phosphorylation (Pressman and Lardy 1956, Lehninger and Remmert 1959, Björntorp et al. 1964, Vázquez-Colón et al. 1966). In view of the known adverse effects of the fatty acids and reactions secondary to the increased formation of fatty acid cyclo-oxygenase (Gaudet and

Levine 1979, Hallenbeck and Furlow 1979) and lipooxygenase products (Murphy et al. 1979, Borgeat and Samuelsson 1979) from arachidonic acid released by phospholipase A₂ activity, it is of interest to note that the accumulation was less pronounced in the 'less vulnerable' cerebellum.

Changes in cyclic nucleotides.

During hypoglycemia, adenosine is formed in large amounts (Chapman et al. 1981) and norepinephrine is released in the cerebral cortex (Agardh et al. 1979). Since both adenosine and norepinephrine have been shown to stimulate cAMP accumulation in cerebral cortical slices (Sattin and Rall 1970), this may explain the increased levels of cAMP in vivo during hypoglycemia. The mechanisms underlying the increase in cGMP levels seem to be different. There is evidence that increased intracellular concentrations of free Ca⁺² (Ferrendelli et al. 1976) in the presence of oxygen (Briggs and DeRubertis 1980) is a major factor regulating cGMP levels in the brain. The oxygen dependence has been ascribed to the fact that hydroperoxide breakdown products of polyenoic fatty acids are the major activators of guanylate cyclase (Asano and Hidaka 1977, Murad et al. 1979) and that such fatty acids are oxidatively degraded following the Ca⁺² stimulated activation of phospholipase A₂. In other words, the changes in cGMP during hypoglycemia may reflect two events of potentially pathophysiological importance: accumulation of free Ca⁺² in intracellular fluids, and breakdown of polyenoic fatty acids to products some of which may induce cellular and vascular damage.

CONCLUSIONS

Hypoglycemia of 30 and 60 min duration caused irreversible neuronal damage in the cerebral cortex, while the alterations in the cerebellum were discrete. The nature and localisation of these changes were different from those occurring during hypoxia/ischemia and previously also described after profound hypoglycemia. It is suggested that the pathogenesis of the two conditions is different.

Markedly reduced levels of energy rich phosphates occurred in both cerebral cortex and cerebellum. However, the energy

failure in the cerebellum appeared after longer periods of hypoglycemia and showed greater variability than in the cerebral cortex. Since the energy stores in the two structures are essentially the same, the glucose supply to the cerebellum must be more efficient.

In spite of the gross energy failure, the CMRO_2 was normal during the first 15 min of hypoglycemia with an abolished EEG activity and only moderately reduced after 30 min. A probable explanation for this is uncoupling of oxidative phosphorylation.

During the same periods, the ratio between 6 times the glucose uptake and the oxygen utilization was reduced, hence substrates other than glucose derived from the blood must have been oxidized. Since there was no evidence that ketone-bodies lactate or pyruvate were transported from the blood to the brain in sufficient quantities, this oxidation must have been upheld at the expense of endogenous substrates. Such endogenous substrates were provided by glycolytic metabolites that were exhausted at the time of onset of isoelectric EEG, and by citric acid cycle intermediates and associated free amino acids, which decreased in both cerebral and cerebellar cortex. Potential substrates were provided by free fatty acids, which accumulated in the cerebral cortex and to a lesser extent in the cerebellar cortex.

Hypoglycemia was associated with a breakdown of phospholipids in the cerebral cortex, but not in the cerebellum. As there was no preferential loss of polyunsaturated fatty acids or ethanolamine phosphoglycerides, rich in such fatty acids, it is concluded that cell damage is not caused by free radical initiated lipid peroxidation.

The mitochondrial function was impaired in vitro. After 30 min of hypoglycemia, the ADP-stimulated respiration was inhibited when NAD-linked, but not FAD-linked substrates were used. There were no signs of uncoupling of oxidative phosphorylation in vitro during hypoglycemia, but evidence of loose coupling with increased state 4 respiration and reduced ADP/O ratios in the recovery period following 60 min of hypoglycemia. Hypoglycemia of 30 min duration was not associated with loss of

phospholipids from the mitochondria.

Energy failure per se cannot alone explain irreversible cell damage (cf. changes in cerebral and cerebellar cortex) but must contribute by initiating a series of metabolic reactions which cause cell dysfunction and death. A tentative suggestion is that when hypoglycemia reaches a certain point, ATP concentration is reduced to below the critical level required to maintain membrane potentials. This leads to increased concentrations of extracellular potassium and spontaneous EEG activity ceases. Energy failure causes increased levels of intracellular free Ca^{+2} , derived from extracellular and intracellular (mitochondria and endoplasmatic reticulum) sites. Increased levels of intracellular Ca^{+2} stimulate phospholipases, leading to increased levels of FFA and breakdown of phospholipids. Accumulation of FFA are known to have several adverse effects on cell function. The release of arachidonic acid in the presence of oxygen favours the synthesis of prostaglandins and leucotrienes which may have deleterious effects on the cell. It seems probable that once increased levels of Ca^{+2} and FFA are at hand, respiratory energy is diverted from oxidative phosphorylation to futile ion cycling, exaggerating the energy failure.

ACKNOWLEDGEMENTS

These studies were carried out at the Laboratory of Experimental Brain Research, University of Lund, Sweden.

I wish to express my warmest thanks to Professor Bo K. Siesjö for his never-flagging encouragement and inspiration.

I am also deeply grateful to Kerstin Beirup for her skilful work with the animals,
and to all the other people working in the laboratory who in one way or another have contributed to this work.

Finally, my grateful thanks to Professor Anders Gustafson and Assistant Professor Bengt Scherstén at the Department of Internal Medicine for their constant support.

The project has been supported by grants from:

Swedish Medical Research Council (Project Nos.14X-263 and B80-12X-03020-11C)

U.S. PHS Grant No.5 R01 NS07838-8 from N.I.H.

Swedish Diabetes Federation

Knut and Alice Wallenberg Foundation

Thorsten and Elsa Segerfalk Foundation

Anna-Lisa and Svend-Erik Lundgren Foundation for Medical Research

Turun Yliopistosäätiö (Turku, Finland)

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CEREBRAL METABOLIC CHANGES IN PROFOUND, INSULIN-INDUCED HYPOGLYCEMIA, AND IN THE RECOVERY PERIOD FOLLOWING GLUCOSE ADMINISTRATION

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(Received 22 March 1978. Revised 22 May 1978. Accepted 6 June 1978)

Abstract—Severe hypoglycemia was induced by insulin in lightly anaesthetized (70% N₂O) and artificially ventilated rats. Brain tissue was frozen *in situ* after spontaneous EEG potentials had disappeared for 5, 10, 15 or 30 min and cerebral cortex concentrations of labile organic phosphates, glycolytic metabolites, ammonia and amino acids were determined. In other experiments, recovery was induced by glucose injection at the end of the period of EEG silence.

All animals with an isoelectric EEG showed extensive deterioration of the cerebral energy state, and gross perturbation of amino acid concentrations. The latter included a 4-fold rise in aspartate concentration and reductions in glutamate and glutamine concentrations to 20 and 5% of control levels respectively. There was an associated rise in ammonia concentration to about $3 \mu\text{mol} \cdot \text{g}^{-1}$.

Administration of glucose brought about extensive recovery of cerebral energy metabolism. For example, after an isoelectric period of 30 min tissue concentrations of phosphocreatine returned to or above normal, the accumulation of ADP and AMP was reversed, there was extensive resynthesis of glycogen and glutamine and full normalisation of tissue concentrations of pyruvate, α -ketoglutarate, GABA and ammonia. However, even after 3 h of recovery there was a reduction in the ATP concentration and thereby in adenine nucleotide pool, moderate elevations of lactate content and the lactate/pyruvate ratio, and less than complete restoration of the amino acid pool. It is concluded that some cells may have been irreversibly damaged by the hypoglycemia.

WHEN SUFFICIENTLY severe, hypoglycemia is accompanied by gross functional derangement, ultimately causing cessation of spontaneous electroencephalographic (EEG) potentials and clinical coma and when sufficiently prolonged, it leads to irreversible neuronal damage. In hypoglycemia that is severe enough to extinguish EEG potentials (or induce coma) there is extensive perturbation of cerebral energy state with decreases in phosphocreatine (PCr) and ATP and increases in ADP and AMP concentrations (TEWS *et al.*, 1965; HINZEN *et al.*, 1971; LEWIS *et al.*, 1974a; NORBERG & SIESJÖ, 1976). It is tempting to assume that cerebral energy failure underlies the cell damage that may occur and this assumption is supported by the finding that hypoglycemic lesions have the same localization to 'selectively vulnerable areas' as occurs in cerebral hypoxia (BRIERLEY *et al.*, 1971; SALFORD *et al.*, 1973). However, some cerebral metabolic changes that occur in hypoglycemia are quite different from those occurring in hypoxia. For example, hypoglycemia is unassociated with cellular acidosis (LEWIS *et al.*, 1974a). Furthermore, when glucose supply is curtailed, oxidative metabolism is supported by endo-

genous substrates, some of which may emanate from cellular structures (ABOOD & GEIGER, 1955; KNAUFF & BÖCK, 1961; HINZEN *et al.*, 1970).

There is extensive information on changes in brain energy, carbohydrate and amino acid metabolism in profound hypoglycemia (for literature, see LEWIS *et al.*, 1974a,b; NORBERG & SIESJÖ, 1976). However, there seems to be only one previous study of recovery following hypoglycemia of sufficient severity to disrupt cerebral energy balance (TEWS *et al.*, 1965). These authors administered glucose to six dogs that had an essentially isoelectric EEG and froze cortical tissue for analyses 11–60 min later. Glucose injection reversed many of the changes that resulted from the hypoglycemia. However, although PCr concentrations were restored and the data indicated rephosphorylation of nucleotides, lactate concentrations rose above control values and there appeared to be a loss of nucleotides. Besides, no clear resynthesis of glycogen or glutamine occurred.

The objective of the present study was to evaluate recovery of cerebral metabolism following pronounced hypoglycemia. To that end, lightly anaesthetized rats were made hypoglycemic with insulin and maintained with an isoelectric EEG for 5, 10, 15 or 30 min. In each group, recovery was induced for 30 min by glucose injection and, following a 30 min isoelectric period, for 90 and 180 min as well.

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METHODS

Animals, operative and sampling techniques. Male Wistar rats (265–380 g) of a S.P.F. Wistar strain (Møllegaard AvelsLaboratorium, Copenhagen) were fasted for 24 h before the experiments but had free access to tap water. One hour before operation they were given an intraperitoneal injection of insulin (Insulin Novo Actrapid®, Novo Industri AB) in a dose of 40 I.U. kg^{-1} . The insulin was dissolved in 0.75 ml of Krebs-Hensleit solution before injection. Control animals were given 0.75 ml of this solution. Anaesthesia was induced with 3% halothane; the animals were then tracheotomized, immobilized with i.v. injection of tubocurarine chloride ($0.5 \text{ mg} \cdot \text{kg}^{-1}$) and ventilated with a Starling type respirator, delivering 70% N_2O and 30% O_2 , to yield an arterial P_{CO_2} of 30–40 mm Hg. Body temperature was maintained close to 37°C . One femoral artery was cannulated for blood pressure recording with an electromanometer and for sampling of blood, and one femoral vein was cannulated for injections. A skin incision was made over the skull bone to accommodate a plastic funnel for later freezing of the brain *in situ* with liquid nitrogen (PONTÉN *et al.*, 1973). The electrocorticogram (EEG) was continuously recorded in all animals from gold-plated copper bolts inserted into the skull bone in the fronto-parietal region (bipolar leads), using an Elema EEG machine.

After the operative procedures had been completed the animals were maintained on 70% N_2O and 30% O_2 . Repeated arterial samples were anaerobically taken from the catheter in glass capillaries for determinations of pH, P_{CO_2} and P_{O_2} , or collected directly in liquid nitrogen for later analyses of glucose.

Experimental groups. There were four series of animals. Series A consisted of four groups ($n = 6$ in each). One was a control group of animals maintained under anaesthesia for 65–150 min before tissue was frozen *in situ*. In this and the other control groups (see below) the duration of anaesthesia was similar to that of the insulin-injected animals. In the other three groups, animals were kept at steady state until the EEG became isoelectric. They were then maintained for either 5, 10 or 15 min with an isoelectric EEG before tissue was sampled. Series B also had four groups ($n = 6$ in each). One control group was maintained under anaesthesia for 130–140 min. Three other groups were allowed a 30 min recovery period, following isoelectric periods of 5, 10 and 15 min respectively. Recovery was induced by i.v. injection of 1.0 ml of a 30% (w/v) glucose solution (in saline). Another 0.5 ml of the same solution was given after 15 min.

In series C there was one control group maintained under anaesthesia for 170 min, one group with an isoelectric EEG record for 30 min, and one which was allowed 30 min of recovery following EEG silence for 30 min ($n = 4$ in each). Recovery was induced as in series B. Series D, finally, consisted of a control group maintained under anaesthesia for 210–270 min and two other groups that were allowed 90 and 180 min of recovery, respectively, following a 30 min period of isoelectric EEG ($n = 4$ in each). Repeated glucose injections were needed during the recovery period.

Analytical techniques. The pH, P_{CO_2} and P_{O_2} in arterial blood were measured immediately after sampling, using microelectrodes operated at 37°C (Radiometer, Copenhagen and Eschweiler & Co, Kiel) with appropriate temperature corrections. Blood and tissue samples were stored at -80°C until analysis. The brains were dissected and

weighed at -20°C . Cerebral cortical tissue from the frontal-parietal regions were extracted at this temperature with HCl-methanol and subsequently with perchloric acid at 0°C . The extracts were neutralized with KOH imidazole base-KCl mixture as described previously (FOLBERGROVÁ *et al.*, 1969). Metabolites were determined with the fluorometric techniques of LOWRY & PASSONNEAU (1972). Analytical conditions for measuring blood glucose concentrations and tissue concentrations of phosphocreatine (PCr), creatine, ATP, ADP, AMP, glycogen, glucose, pyruvate, lactate, α -ketoglutarate (α -KG), glutamate, glutamine, aspartate, alanine, GABA and ammonia (NH_4^+) have been previously described (FOLBERGROVÁ *et al.*, 1972a,b; 1974a,b).

The tissue samples used for analysis of cyclic nucleotides were extracted at -20°C with HCl-methanol and subsequently with perchloric acid at 0°C . For the measurements of cyclic AMP the extract was neutralized with KOH imidazole base-KCl mixture as described above. For the measurements of cyclic GMP an acidic extract was prepared as described by LOWRY & PASSONNEAU (1972). KHCO_3 and acetate-buffer (pH 4.6) were added, pH 4.6–5.0. Cyclic GMP was assayed using the radioimmunoassay described by STEINER *et al.* (1972) using commercial kit (Cyclic GMP (^{125}I -labelled) RIA kit, New England Nuclear, Boston, U.S.A. Cyclic AMP was measured with a protein-binding technique, using Cyclic AMP Assay kit, Amersham, England.

Calculation and statistics. The 'energy state' of the tissue was calculated in terms of the adenylate energy charge (E.C.) according to ATKINSON (1968). Statistical differences were evaluated with Student's *t*-test. The following symbols are used: * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$.

RESULTS

Since the objective of the study was to evaluate cerebral metabolic changes due to severe hypoglycemia it was essential to exclude an influence of systemic variables. Table 1 shows that neither blood pressure nor arterial P_{O_2} fell during the period of isoelectric EEG, or in the recovery period following glucose injection. Arterial P_{CO_2} was between 32 and 37 mm Hg in all groups and body temperature was within 0.2°C of control. There was a moderate fall in arterial pH in the 30 min groups. Comparable results were obtained in animals allowed recovery periods of 90 or 180 min.

Events occurring during hypoglycemia

In all animals, the period of severe hypoglycemia was defined in terms of duration of period of abolished EEG activity. The beginning of the period of severe hypoglycemia was defined as the point when spontaneous EEG activity disappeared. During the period of electrical silence (5, 10, 15 and 30 min) no spontaneous EEG activity was observed.

In each experiment the EEG pattern was correlated to blood glucose concentrations. In some, blood glucose was determined with intervals of about 30 min following completion of operative procedures (about 75–80 min after insulin injection). Figure 1 shows blood glucose concentrations in control animals and in 9 representative animals injected with insulin. In

TABLE 1. PHYSIOLOGICAL PARAMETERS DURING INSULIN-INDUCED HYPOGLYCEMIA AND IN THE RECOVERY PERIOD FOLLOWING GLUCOSE INJECTION

Group	Period of EEG silence (min)	No. of animals	MABP (mm Hg)	Pa _{O₂} (mm Hg)	Pa _{CO₂} (mm Hg)	pH	Temp. (°C)
Controls		10	155 ± 7	122 ± 4	37.3 ± 1.2	7.348 ± 0.012	37.1 ± 0.1
Insulin-injected	5	6	139 ± 7	129 ± 13	31.7 ± 1.7	7.370 ± 0.053	37.2 ± 0.2
	10	6	169 ± 9	124 ± 12	33.9 ± 1.1	7.392 ± 0.014	37.3 ± 0.1
	15	6	158 ± 15	137 ± 12	33.9 ± 2.2	7.375 ± 0.023	37.2 ± 0.1
	30	4	130 ± 11	134 ± 4	37.8 ± 3.3	7.268 ± 0.029	37.1 ± 0.3
Controls		10	149 ± 6	127 ± 5	36.1 ± 1.1	7.357 ± 0.013	37.3 ± 0.1
Recovery (30 min)	5	6	148 ± 5	127 ± 12	35.0 ± 0.5	7.377 ± 0.011	37.5 ± 0.1
	10	6	153 ± 7	132 ± 11	35.0 ± 1.4	7.360 ± 0.020	37.5 ± 0.2
	15	6	135 ± 6	127 ± 9	34.9 ± 2.1	7.356 ± 0.036	37.3 ± 0.2
	30	4	138 ± 8	129 ± 15	34.2 ± 0.8	7.279 ± 0.037	37.3 ± 0.1

The values are means ± S.E.M. MABP = mean arterial blood pressure.

control animals, there was a gradual rise in blood glucose concentration. Results obtained after insulin administration showed two typical features. First, an isoelectric EEG was obtained after periods varying between 113 and 252 min (mean 170 min) following insulin injection. Second, spontaneous EEG activities disappeared when blood glucose concentrations decreased to values around or below $1 \mu\text{mol} \cdot \text{g}^{-1}$ (c.f. LEWIS *et al.*, 1974b). During the isoelectric periods, no value was higher than $1.39 \mu\text{mol} \cdot \text{g}^{-1}$ and the lowest was $0.17 \mu\text{mol} \cdot \text{g}^{-1}$ (mean ± S.E.M. of 85 determinations was 0.77 ± 0.03).

Table 2 shows changes in cerebral metabolites after 5, 10, 15 and 30 min of isoelectric EEG. For the period 5–15 min the results confirm those previously reported (NORBERG & SIESJÖ, 1976). Thus, there was extensive breakdown of PCR and ATP, with accumulation of ADP and AMP, reductions in concentrations of glycolytic metabolites and of α -KG, massive accumulation of ammonia, and amino acid changes consisting of reductions in concentrations of glutamate, glutamine, GABA and alanine, as well as of a pronounced rise in aspartate concentration. After 30 min of isoelectric EEG changes in labile phos-

phates, carbohydrate metabolites and amino acids were further exaggerated. For example, ATP concentrations fell to $0.5 \mu\text{mol} \cdot \text{g}^{-1}$ and those of glutamate and glutamine to 2.8 and $0.3 \mu\text{mol} \cdot \text{g}^{-1}$ respectively. In this group, the sum of adenine nucleotides fell to 63% of control, and the sum of amino acids to 78% of control. The results confirm those previously published (LEWIS *et al.*, 1974a) in showing a decreased lactate/pyruvate ratio, probably reflecting oxidation of cellular redox systems.

Events during recovery

During recovery most animals had blood glucose concentrations in the range of $3\text{--}10 \mu\text{mol} \cdot \text{g}^{-1}$. However, values gradually fell, necessitating repeated injections (every 15 min). In this way, no single value fell below $2.25 \mu\text{mol} \cdot \text{g}^{-1}$.

All animals regained some spontaneous EEG activity whether EEG was isoelectric for 5, 10, 15 or 30 min. With the shorter periods of EEG silence EEG reappeared sooner and seemed to show a more extensive recovery at the end of the 30 min recovery period. However, even after 180 min of recovery the EEG had not normalized but showed a slow wave pattern.

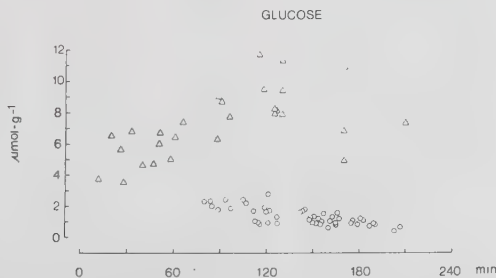


FIG. 1. Time course of changes in blood glucose concentrations in control animals (Δ) and in insulin-injected animals ($\circ$). For control animals, time scale denotes time (in min) after induction of anaesthesia and, for insulin-injected animals, time after injection of insulin.

TABLE 2. CEREBRAL CORTICAL CONCENTRATIONS OF LABILE PHOSPHATES, CARBOHYDRATE SUBSTRATES, AMINO ACIDS AND AMMONIA DURING INSULIN-INDUCED HYPOGLYCEMIA

	Isoelectric EEG (min)				
	Control (n = 10)	5 (n = 6)	10 (n = 6)	15 (n = 6)	30 (n = 4)
PCr	4.44 ± 0.08	0.68 ± 0.07	0.50 ± 0.07	0.60 ± 0.05	0.29 ± 0.03
Cr	5.78 ± 0.12	9.27 ± 0.21	9.55 ± 0.11	9.61 ± 0.15	9.50 ± 0.18
ATP	3.00 ± 0.03	1.14 ± 0.06	0.98 ± 0.08	0.97 ± 0.06	0.53 ± 0.04
ADP	0.280 ± 0.007	1.042 ± 0.077	0.980 ± 0.053	0.921 ± 0.042	1.014 ± 0.038
AMP	0.038 ± 0.006	0.485 ± 0.039	0.499 ± 0.041	0.462 ± 0.032	0.535 ± 0.047
Glycogen	2.38 ± 0.09	0.06 ± 0.01	0.05 ± 0.01	0.04 ± 0.01	0.03 ± 0.01
Glucose	2.94 ± 0.28	0.09 ± 0.02	0.09 ± 0.02	0.11 ± 0.02	0.02 ± 0.02
Lactate	1.45 ± 0.10	0.29 ± 0.03	0.21 ± 0.01	0.27 ± 0.05	0.13 ± 0.03
Pyruvate	0.117 ± 0.007	0.026 ± 0.003	0.027 ± 0.001	0.029 ± 0.004	0.029 ± 0.004
Lac/Py	12.5 ± 0.6	12.7 ± 2.2 ^{NS}	7.7 ± 0.7	9.6 ± 1.7 ^{NS}	4.3 ± 0.4
α-KG	0.135 ± 0.011	0.039 ± 0.001	0.035 ± 0.004	0.028 ± 0.003	0.012 ± 0.003
Glutamate	12.00 ± 0.16	6.72 ± 0.86	4.21 ± 0.34	4.47 ± 0.18	2.79 ± 0.16
Aspartate	3.52 ± 0.11	12.96 ± 0.85	15.28 ± 0.55	15.21 ± 0.44	13.74 ± 0.60
Glutamine	5.08 ± 0.12	1.57 ± 0.38	0.97 ± 0.16	0.91 ± 0.12	0.29 ± 0.09
Alanine	0.448 ± 0.019	0.317 ± 0.027*	0.251 ± 0.011	0.300 ± 0.028	0.253 ± 0.016
GABA	2.00 ± 0.04	1.78 ± 0.06	1.51 ± 0.09	1.50 ± 0.17	0.97 ± 0.03
NH ₄ ⁺	0.37 ± 0.04	3.94 ± 0.17	3.81 ± 0.15	3.70 ± 0.36	3.06 ± 0.16

The values are given in $\mu\text{mol}\cdot\text{g}^{-1}$ wet weight and represent means $\pm$ S.E.M. $P < 0.01$ for all values except those marked NS (not significant), and * = $P < 0.05$.

Table 3 shows cerebral metabolic concentrations as measured after 30 min of recovery following isoelectric periods of 5, 10, 15 and 30 min respectively. PCr concentrations were restored to normal, or super-normal concentrations, with reciprocal changes in creatine concentrations. The ADP and AMP concentrations were close to normal but ATP concentration was reduced in proportion to the duration of EEG

silence. However, since the fall in energy charge following 15 and 30 min of isoelectric EEG was moderate (from 0.946 ± 0.003 to 0.936 ± 0.002 and 0.918 ± 0.005 respectively) the reduction in ATP concentration mainly reflected loss of adenine nucleotides.

There was extensive restoration of tissue glucose concentrations but little resynthesis of glycogen. In

TABLE 3. CEREBRAL CORTICAL CONCENTRATIONS OF LABILE PHOSPHATES, CARBOHYDRATE SUBSTRATES, AMINO ACIDS AND AMMONIA IN THE RECOVERY PERIOD FOLLOWING GLUCOSE INJECTION AFTER INSULIN-INDUCED HYPOGLYCEMIA

	Isoelectric EEG (min) followed by recovery 30 min				
	Control (n = 10)	5 (n = 6)	10 (n = 6)	15 (n = 6)	30 (n = 4)
PCr	4.46 ± 0.05	4.67 ± 0.13	4.62 ± 0.17	4.74 ± 0.19	4.97 ± 0.12**
Cr	5.97 ± 0.14	5.53 ± 0.13	5.47 ± 0.34	5.80 ± 0.34	5.36 ± 0.16
ATP	2.96 ± 0.03	2.58 ± 0.05***	2.49 ± 0.06***	2.41 ± 0.04***	2.05 ± 0.09***
ADP	0.280 ± 0.007	0.263 ± 0.007	0.258 ± 0.010	0.269 ± 0.009	0.297 ± 0.020
AMP	0.036 ± 0.006	0.035 ± 0.003	0.045 ± 0.004	0.040 ± 0.004	0.046 ± 0.009
Glycogen	2.47 ± 0.10	0.36 ± 0.04***	0.39 ± 0.08***	0.22 ± 0.02***	0.23 ± 0.06***
Glucose	2.75 ± 0.29	1.37 ± 0.22*	1.86 ± 0.14	1.99 ± 0.22	2.85 ± 0.55
Lactate	1.45 ± 0.09	1.71 ± 0.20	2.35 ± 0.54	2.59 ± 0.49	2.66 ± 0.49
Pyruvate	0.120 ± 0.007	0.102 ± 0.007	0.120 ± 0.011	0.121 ± 0.013	0.161 ± 0.020
Lac/Py	12.2 ± 0.5	16.5 ± 1.2*	18.9 ± 2.7	20.9 ± 1.5***	16.2 ± 1.5*
α-KG	0.130 ± 0.008	0.098 ± 0.009*	0.103 ± 0.005*	0.098 ± 0.012*	0.115 ± 0.017
Glutamate	11.93 ± 0.13	11.58 ± 0.15	11.63 ± 0.15	11.01 ± 0.22*	10.22 ± 0.25**
Aspartate	3.55 ± 0.09	4.24 ± 0.15*	3.84 ± 0.11	3.98 ± 0.15	3.11 ± 0.21
Glutamine	5.40 ± 0.32	3.37 ± 0.36**	3.14 ± 0.25**	2.39 ± 0.17***	2.12 ± 0.26***
Alanine	0.474 ± 0.025	0.710 ± 0.066*	0.715 ± 0.060**	0.768 ± 0.031***	0.914 ± 0.061***
GABA	2.04 ± 0.05	2.09 ± 0.10	2.10 ± 0.08	2.21 ± 0.09	1.88 ± 0.03
NH ₄ ⁺	0.35 ± 0.01	0.31 ± 0.04	0.31 ± 0.02	0.32 ± 0.04	0.25 ± 0.01**

The values are given in $\mu\text{mol}\cdot\text{g}^{-1}$ wet weight and represent means $\pm$ S.E.M. * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$.

most groups α -KG concentrations remained slightly depressed but pyruvate concentrations were restored. Interestingly enough, there was a tendency towards elevation of lactate concentrations and lactate/pyruvate ratios were increased.

Ammonia concentrations returned to normal or even subnormal (30 min group) concentrations. Of the amino acids, only aspartate and GABA concentrations returned to normal or near-normal values, while alanine was clearly elevated and, at least in the 15 and 30 min groups, glutamate and glutamine concentrations remained low. As a result there was a decrease in amino acid pool, roughly proportional to the duration of the preceding EEG silence.

Time-dependent recovery of cerebral metabolism was studied after 30 min of EEG silence. The results are given in Figs. 2-4 which, to facilitate inspection of results, also contain data for the hypoglycemic periods (taken from Table 2). Figure 2 shows PCr and ATP concentrations, sum of adenine nucleotides, and adenylate energy charge. PCr rose to values exceeding control and the energy charge returned to normal values ($P > 0.05$) after 180 min. However, the ATP concentration and the adenine nucleotide pool remained depressed and no synthesis of adenine nucleotides occurred between 90 and 180 min. As a result, there was a lingering reduction of pool size by about 20%.

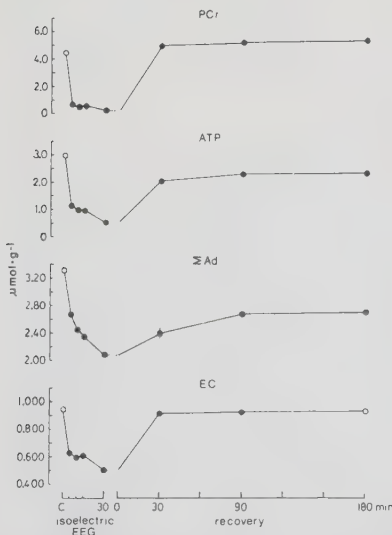


FIG. 2. Cerebral cortical concentrations of PCr, ATP, sum of adenine nucleotides (Σ Ad) and adenylate energy charge (E.C.) after 5, 10, 15 or 30 min of isoelectric EEG and after 30, 90 or 180 min of recovery. Filled symbols indicate values significantly ($P < 0.05$) different from the controls (C). The results are means. S.E.M. is given as vertical bars, if larger than symbol.

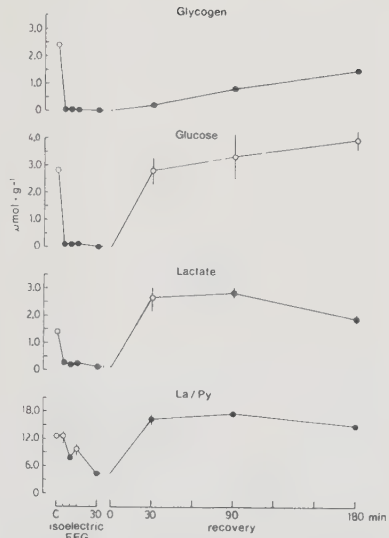


FIG. 3. Cerebral cortical concentrations of glycogen, glucose, lactate and lactate/pyruvate ratio after 5, 10, 15 or 30 min of isoelectric EEG and after 30, 90 or 180 min of recovery. Filled symbols indicate values significantly ($P < 0.05$) different from the controls (C). The results are means. S.E.M. is given as vertical bars, if larger than symbol.

Figure 3 gives changes in glycolytic metabolites. In the recovery period glucose concentrations were normal or elevated. There was a linear rise in glycogen concentration at a rate of about $0.01 \mu\text{mol} \cdot \text{g}^{-1} \text{min}^{-1}$ but, after 180 min, the value was still lower than control ($P < 0.01$).

Both lactate concentration and the lactate/pyruvate ratio remained elevated in the recovery period although there was a tendency toward normalization between 90 and 180 min.

Ammonia and amino acid concentrations are illustrated in Fig. 4. Ammonia concentrations fell at 30 and 90 min but the value measured at 180 min was not significantly different from control, possibly because one value ($0.38 \mu\text{mol} \cdot \text{g}^{-1}$) was considerably higher than the others.

GABA concentrations returned to normal values, but alanine remained elevated (cf. lactate concentrations). There was a progressive rise in glutamine concentration and, after 180 min, the values were not significantly lower than control. However, there was a lingering reduction in glutamate and a small decrease in aspartate concentration (at 90 and 180 min). As a net result of the amino acid changes the amino acid pool size was reduced by $3.2 \mu\text{mol} \cdot \text{g}^{-1}$ at 90 min, and by $2.6 \mu\text{mol} \cdot \text{g}^{-1}$ at 180 min. Thus, complete resynthesis of amino acids did not occur during recovery.

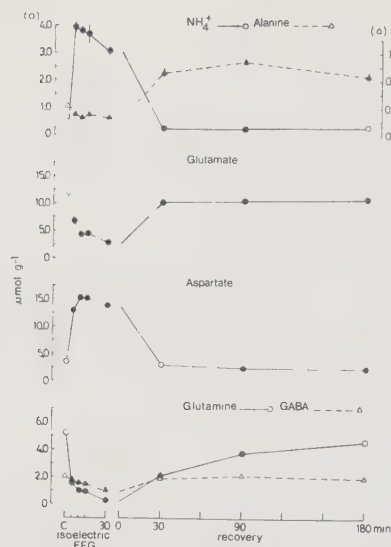


FIG. 4. Cerebral cortical concentrations of ammonia (NH_4^+), alanine, glutamate, aspartate, glutamine and GABA after 5, 10, 15 or 30 min of isoelectric EEG and after 30, 90 or 180 min of recovery. Filled symbols indicate values significantly ($P < 0.05$) different from the controls (C). The results are means. S.E.M. is given as vertical bars, if larger than symbol.

Changes in cyclic nucleotides

Since previous results have shown that hypoglycemia leads to changes in cyclic GMP (GORELL *et al.*, 1976), concentrations of cyclic nucleotides were measured in the hypoglycemic groups, and during recovery. Figure 5 shows that the concentrations of cyclic GMP and cyclic AMP increased during severe hypoglycemia, but the change in cyclic AMP disappeared after 30 min.

During recovery, concentrations of both cyclic nucleotides normalized. After 180 min of recovery, cyclic AMP was 1.41 ± 0.04 and cyclic GMP 0.031 ± 0.002 $\mu\text{mol} \cdot \text{kg}^{-1}$, respectively (means $\pm$ S.E.M., $n = 4$ in each group).

DISCUSSION

As remarked in the introduction, there are few previous studies of recovery of cerebral metabolism following hypoglycemia. With short periods of insulin-induced coma there is full restoration of cerebral blood flow (CBF) and oxygen consumption (CMRO_2) in man (DELLA PORTA *et al.*, 1964). However, if sufficiently severe and sufficiently prolonged hypoglycemia leads to irreversible clinical coma with markedly reduced CMRO_2 (FAZEKAS *et al.*, 1951), and to neur-

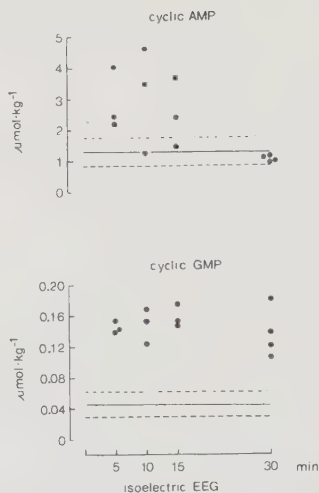


FIG. 5. Individual values of cyclic AMP and cyclic GMP after 5, 10, 15 and 30 min of isoelectric EEG. Horizontal solid line represent the mean, and the interrupted lines are ± 2 S.D. of controls ($n = 7$).

onal damage (MEYER, 1963). There are no clinical results defining the duration of hypoglycemia that leads to cell damage but experimental results have shown that blood glucose concentrations must fall to values below $1\text{--}2$ $\mu\text{mol} \cdot \text{g}^{-1}$, and damage was observed in experiments in which somatosensory evoked potentials were abolished for 49–92 min (BRIERLEY *et al.*, 1971).

Comparable studies of cerebral metabolite levels are few. Recovery of cerebral concentrations of some carbohydrate metabolites and amino acids has been studied in mice (GORELL *et al.*, 1976; 1977) but in these experiments the hypoglycemia was not sufficiently severe to alter concentrations of PCr and ATP. More relevant to the present discussion are the results of TEWS *et al.* (1965). As stated, these showed extensive recovery of cerebral metabolite concentrations. However, the results gave no hint to the extent of final recovery, and the method available did not allow conclusions on recovery of cerebral energy state.

The objective of the present study was to evaluate recovery of cerebral energy metabolism following periods of hypoglycemia 'coma' that could critically affect viability of brain cells. Our previous (LEWIS *et al.*, 1974a, b; NORBERG & SIESJÖ, 1976) and present results show that when spontaneous EEG activity disappears there is extensive deterioration of cerebral energy state.

Events during hypoglycemia

The present results obtained during hypoglycemia (5–15 min of EEG silence) corroborate those pre-

viously published (NORBERG & SIESJÖ, 1976). Those obtained after 30 min of isoelectric EEG show that there is further deterioration of cerebral energy state, and further reductions in tissue concentrations of glutamate, glutamine, GABA and alanine, with no additional elevation of aspartate levels. As a result, the sum of amino acids decreased by $5 \mu\text{mol} \cdot \text{g}^{-1}$, probably reflecting mobilization of carbon skeleton by oxidative deamination. Thus, the loss of amino acids (or of adenine nucleotides) cannot be ascribed to unspecific loss of substances across leaky membranes since the sum of PCr and creatine did not fall.

The results obtained on cyclic GMP concentrations corroborate those of GORELL *et al.* (1976) in showing elevated cerebral cortex levels in hypoglycemia, and they show that the accumulation persists in hypoglycemic 'coma' of maximally 30 min duration. As suggested by GORELL *et al.* (1976) the elevated cyclic GMP levels may possibly reflect altered activity at cholinergic synapses, a suggestion which is in line with the report of GIBSON & BLASS (1976) that hypoglycemia interferes with acetylcholine synthesis. Our results show that cyclic AMP concentrations rise during the first 15 min of EEG silence but that they return to control values after 30 min. Tentatively, accumulation of cyclic AMP reflects enhanced activity in catecholaminergic pathways while the subsequent return of the cyclic AMP concentration to control values may be caused by lack of substrate (ATP). This hypothesis receives support from results showing that severe hypoglycemia is accompanied by increased synthesis of catecholamines (AGARDH *et al.*, in preparation).

Events in recovery period

In corroboration of results reported by TEWS *et al.* (1965) the present ones demonstrate extensive recovery of cerebral metabolite levels after extended periods of hypoglycemic 'coma'. Most important, the results show restitution of tissue concentrations of PCr, ADP and AMP, suggesting extensive recovery of mitochondrial metabolism. After long periods of EEG silence, PCr concentrations rose above control values. Since the creatine kinase reaction is freely reversible the rise in PCr/creatine ratio either must reflect changes in the compartmental concentrations in reactants, an increase in intracellular pH, or a change in the equilibrium constant which is Mg^{2+} -dependent (KUBY & NOLTMANN, 1962; see also SIESJÖ *et al.*, 1972). Although the adenylate energy charge eventually returned to control values, following administration of glucose, there was a persisting decrease in sum of adenine nucleotides. The loss of nucleotides during hypoglycemia is easily explained by deamination and dephosphorylation of AMP with subsequent translocation of breakdown products from tissue to blood (e.g. DEUTICKE *et al.*, 1966; McILWAIN, 1972). Resynthesis of nucleotides can either occur by *de novo* synthesis or by salvage pathways, utilizing precursors that have been retained

within the tissue or transported across the blood brain barrier (e.g. KLIJHUIS *et al.*, 1974; PARDRIDGE & OLDENDORF, 1977). Whether resynthesis occurs by *de novo* or salvage pathways the process is slow, and a lingering reduction in nucleotide pool size does not necessarily reflect an irreversible event.

The near-complete recovery of cerebral energy metabolism is also evident from the fact that extensive resynthesis occurred of tissue concentration of glycogen and glutamine, two ATP-requiring reactions, and that the glutamate/aspartate ratio normalized. However, full recovery of amino acid metabolism did not occur within 180 min since alanine concentration remained elevated and the sum of amino acids was subnormal.

In summary, although the present results have shown that extensive recovery of cerebral metabolism occurs after extended periods of severe hypoglycemia (with abolition of EEG potentials) the results are compatible with a certain degree of neuronal damage.

Acknowledgements—The skilful technical assistance of KERSTIN BEIRUP, BARBRO ASPLUND, KARIN HANSSON, GERTIE JOHANSSON and LENA SJÖBERG is gratefully acknowledged. This study was supported by grants from the Swedish Medical Research Council (Project No. 14X-263), from U.S. PHS Grant No. 5 R01 NS07838-8 from N.I.H., and the Swedish Diabetes Federation.

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Endogenous Substrates Utilized by Rat Brain in Severe Insulin-Induced Hypoglycemia

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Abstract: Several previous studies have demonstrated that severe hypoglycemia is accompanied by consumption of endogenous brain substrates (glycolytic and citric acid cycle metabolites and free amino acids) and some have shown a loss of structural components as well, notably phospholipids. In the present study, on paralysed and artificially ventilated rats, we measured cerebral oxygen and glucose consumption during 30 min of hypoglycemic coma (defined as hypoglycemia of sufficient severity to cause cessation of spontaneous EEG activity) and calculated the non-glucose oxygen consumption. In an attempt to estimate the missing substrate we measured tissue concentrations of phospholipids and RNA.

After 5 min of hypoglycemic coma, tissue phospholipid content decreased by about 8% with no further change during the subsequent 55 min. A similar reduction remained after 90 min of recovery, induced by glucose administration following 30 min of coma. Since no preferential loss of polyenoic fatty acids or of ethanolamine phosphoglycerides occurred, it is concluded that loss of phospholipids was due to phospholipase activity rather than to peroxidative degradation. The free fatty acid concentration increased sixfold after 5 min of coma and remained elevated during the course of hypoglycemia. A 9% reduction in tissue RNA content was observed after 30 min of hypoglycemia.

Calculations indicated that available endogenous carbohydrate and amino acid substrates were essentially consumed after 5 min of coma, and that other non-glucose substrates must have accounted for approximately $50 \mu\text{mol} \cdot \text{g}^{-1}$ of oxygen ($8.3 \mu\text{mol} \cdot \text{g}^{-1}$ in terms of glucose equivalents) within the 5–30 min period. The 10% reduction in phospholipid-bound fatty acids was more than sufficient (in four- to fivefold excess) to account for this oxygen consumption. However, since no further degradation occurred in the 5–30 min period, there is no simple, direct, quantitative relationship between oxygen consumption and cortical fatty acid oxidation during this interval. The possibility thus remains that unmeasured exogenous or endogenous substrates were utilized. **Key Words:** Hypoglycemia—Rat brain—Phospholipids—Fatty acids—Amino acids—Glucose. Agardh C.-D. et al. Endogenous substrates utilized by rat brain in severe insulin-induced hypoglycemia. *J. Neurochem.* 36, 490–500 (1981).

In man, insulin-induced hypoglycemia leads to a decrease in cerebral glucose consumption (CMR_{gl}), but since the oxygen consumption (CMR_{O_2}) is either normal or only moderately reduced (Kety et al., 1948; Eisenberg and Seltzer, 1962; Della Porta

et al., 1964; Gottstein and Held, 1967) alternative substrates must be oxidized. Animal experiments have shown that, in severe hypoglycemia, exogenous glucose accounts for less than 50% of the oxygen consumed (Pappenheimer and Setchell, 1973;

Received March 27, 1980; revised July 11, 1980; accepted July 22, 1980.

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Abbreviations used: BHT, 2,6-Di-*tert*-butyl-*p*-cresol; FA, Fatty acid; FFA, Free fatty acid; β -HB, β -Hydroxybutyrate; AcAc, Acetoacetate; CPG, Choline phosphoglycerides; IPG + SPG, Inositol plus serine phosphoglycerides; EPG, Ethanolamine phosphoglycerides.

Norberg and Siesjö, 1976), and since such oxygen/glucose ratios may persist for periods exceeding 2 h (Pappenheimer and Setchell, 1973), large amounts of alternative substrates are required.

Under certain circumstances the brain can extract and oxidize ketone bodies and lactate (see Sokoloff et al., 1977). However, these substrates are utilized in proportion to their arterial concentrations and since these are low in insulin-induced hypoglycemia, it seems less probable that the alternative substrates are exogenous. The nature of the endogenous substrates utilized is only partly known. Readily available substrates are provided by glycogen, glucose, glycolytic metabolites, citric acid cycle intermediates, and free amino acids (Cravioto et al., 1951; Goldberg et al., 1966; Lewis et al., 1974; Norberg and Siesjö, 1976). However, since the major part of this substrate pool is utilized during the first 5 min of hypoglycemic coma (Norberg and Siesjö, 1976; Agardh et al., 1978), additional substrates must be mobilized.

Although experiments with artificially perfused cat brains indicated that exclusion of exogenous substrate was accompanied by breakdown of proteins, nucleic acids, and lipids in subcellular fractions of brain tissue (Abood and Geiger, 1955), other results on intact animals rendered hypoglycemic by insulin failed to reveal a significant breakdown of proteins or nucleic acids (Samson et al., 1959; Knauff et al., 1961; see also Dawson and Richter, 1950). There is evidence, though, that breakdown of phospholipids can provide the missing substrate. Thus, although 2 groups failed to detect significant changes in phospholipid content (Page et al., 1931; Petersen and Schou, 1955) others noted that prolonged and/or severe hypoglycemia is accompanied by a significant reduction in phospholipid phosphorus or in individual phospholipid concentrations (Randall, 1940; McGhee et al., 1951; Knauff et al., 1961; Hinzen et al., 1970). In these studies, the phospholipid content of the brain was reduced by 5–15% of control.

In the present study we have tried to correlate the 'non-glucose' oxygen consumption of the brain in severe hypoglycemia to the decrease in the phospholipid content of the tissue. A special emphasis was placed on time-dependent changes in $CMRO_2$ and substrate utilization in coma, i.e., during the period in which spontaneous EEG activity had ceased. Since histopathologic studies have indicated that hypoglycemia of that severity is accompanied by partial disappearance of endoplasmic reticulum in neurons (Kalimo et al., 1980), we analysed both phospholipid and RNA content in cerebral cortical tissue.

Some of the results concerning the changes in free fatty acid concentrations have been given previously in a preliminary report (Agardh et al., 1980b).

METHODS

Animals, Operative and Sampling Techniques

The experiments were performed on male S.P.F. Wistar rats weighing 260–435 g (Møllegaard Avlslaboratorium, Copenhagen). The animals were fasted overnight but had free access to water. One hour before operation insulin (Insulin Novo Actrapid®, Novo Industries A/S) in a dose of 40 I.U. $\cdot$ kg⁻¹ was injected i.p. The insulin was dissolved in 0.75 ml of Krebs-Hensleit solution before injection. Control animals were given 0.75 ml of this solution. Anaesthesia was induced with 2–3% halothane; the animals were tracheotomized, immobilized with tubocurarine chloride (0.5 mg $\cdot$ kg⁻¹ i.v.), connected to a Starling type respirator, and ventilated on 1% halothane and 70% N₂O. Body temperature was maintained between 36.5 and 37.5°C. Both femoral arteries were cannulated for continuous blood pressure recording with an electromanometer and for sampling of blood, and one femoral vein was cannulated for injections. The electrocorticogram (EEG) was continuously recorded in all animals from gold-plated copper bolts inserted into the skull bone in the fronto-parietal region (bipolar leads), using an Elema EEG machine. In one series (series A) a small skin incision was made over the skull bone, and a burr hole made over the distal part of the superior sagittal sinus for sampling of cerebral (cortical) venous blood. In another series (series B) one retroglenoidal vein was cannulated for sampling of cerebral venous blood as previously described (Nilsson and Siesjö, 1976; 1980). In the animals used for analysis of lipids and ribonucleic acid (RNA) (series C and D), a skin incision was made over the skull bone to accommodate a plastic funnel for later freezing of the brain *in situ* with liquid nitrogen (Pontén et al., 1973).

After the operative procedures had been completed, the animals were given heparin i.v. and were ventilated on 70% N₂O and 30% O₂ to yield an arterial P_{CO_2} of 30–40 mm Hg and a P_{O_2} around 100 mm Hg. Repeated arterial samples were anaerobically taken from the catheter in glass capillaries for determination of pH, P_{CO_2} and P_{O_2} , or collected directly in liquid nitrogen for later analyses of glucose and ketone bodies.

Experimental Groups

There were 4 series of animals.

Series A. Measurement of $CMRO_2$

Series A consisted of 2 groups. The animals were ventilated until spontaneous EEG activity disappeared (in the following, termed isoelectric EEG). They were then maintained for either 15 or 30 min with an isoelectric EEG before measurement of CBF ($n = 4$ in each). CBF was measured by a modification (Norberg and Siesjö, 1976; Berntman et al., 1979) of the Kety and Schmidt technique (1948). The brain was saturated by ¹³³Xenon (¹³³Xe) added to the inspired gas for 20 min. At the end of this period samples were taken from artery and vein (superior sagittal sinus) for determination of total oxygen content (TO_2) and ¹³³Xe activity. Thereafter, the ¹³³Xe source was disconnected and repeated arterial and venous samples were

taken for ^{133}Xe activity determination during the desaturation period. About 1 min after the start of desaturation a second pair of arterial and venous samples were taken for To_2 determination. CMRo_2 was calculated by multiplying the CBF with the mean of the A-V differences in To_2 (AVDO_2). The arterial blood pressure was kept constant during the sampling period by infusion of donor blood from a hypoglycemic rat. No experiment was considered valid unless the AVDO_2 samples differed by less than 10% from the mean value.

Series B. Measurements of Arterio-Venous Differences for Glucose and Oxygen

Paired samples of arterial and cerebral venous blood were taken repeatedly in 6 rats for determination of oxygen and glucose content and calculation of cerebral AVDO_2 and AVD_{gl} . These animals were prepared for continuous cerebral venous outflow measurements according to the method of Nilsson and Siesjö (1980). The preparation involves cannulation of one retroglacial vein, the contralateral retroglacial vein being occluded, and measurement of the venous outflow via a closed drop recording circuit returning the blood through a catheter in the superior caval vein. Cerebral venous blood was sampled via a thin needle inserted in the retroglacial vein catheter permitting flow in the circuit to continue and pass the tip of the needle during sampling. In 4 animals the paired samples for oxygen and glucose determination were taken in immediate sequence. In 2 animals a system of sampling capillaries was arranged in order to permit determination of oxygen and glucose content in fractions of the same portion of blood. The total oxygen content was determined immediately, whereas samples for analysis of glucose content were frozen in liquid nitrogen and stored at -80°C until analysed. Some of these samples were also used for determination of ketone bodies.

Series C. Tissue Measurements of Lipids

Series C consisted of 6 groups with 6 animals in each. One was a control group kept under anaesthesia for 200 min before frozen *in situ*. In this group, one sample for analysis of total fatty acids was lost and this group therefore consisted of 5 animals. In 4 other groups, animals were ventilated until the EEG became isoelectric. They were then maintained for either 5, 15, 30, or 60 min with an isoelectric EEG before tissue was sampled. In another group recovery was induced after a 30-min period with isoelectric EEG by an i.v. injection of 0.5 ml 50% (w/v) glucose followed by a slow i.v. infusion of the glucose solution ($1 \text{ ml} \cdot \text{h}^{-1}$) for 90 min before tissue was frozen *in situ*.

Series D. Tissue Measurements of RNA

In series D there was one control group ($n = 5$) kept under anaesthesia for 193 ± 8 min (mean $\pm$ s.d.) and one group with an isoelectric EEG-recording for 30 min ($n = 6$) before tissue was frozen *in situ*.

Analytical Technique

The pH, PCO_2 , and Po_2 in arterial blood were measured immediately after sampling, using microelectrodes operated at 37°C (Radiometer, Copenhagen and Eschweiler & Co., Kiel) with appropriate temperature corrections. Blood and tissue samples were stored at -80°C until analysis. Arterial and cerebral To_2 content were measured by a polarographic technique (Borgström et al., 1974). The ^{133}Xe activity was measured by gamma counting. The brains were dissected and weighed at -20°C for analysis of RNA and lipids.

Total cortical RNA was extracted and analysed according to a modified Schmidt-Thannhauser procedure (Schmidt and Thannhauser, 1945; Munro and Fleck, 1966). Acid-soluble metabolites were extracted from frozen cortex as described by Folbergrová et al. (1972a). Lipids were removed from the acid-insoluble precipitate by sequential washes with cold water, ethanol, ethanol-ether (1:1) twice, and ether (twice). The RNA in the remaining precipitate was hydrolysed by an overnight incubation with 2.5 ml 0.3 M-KOH at 37°C . The extract was cooled in ice and DNA and protein precipitated out with 1 ml 1 M- HClO_4 . An aliquot of the supernatant fraction was neutralized by the addition of KOH. The concentration of the RNA hydrolysate in the extract was determined by measuring the ultraviolet absorbance at 260 nm and 280 nm. The results were checked by also determining the P content in the extract (Itaya and Ui, 1966; Belfrage et al., 1970). The two methods gave comparable results. For the calculations of the RNA content, an average extinction coefficient at 260 nm = 11.0 mM^{-1} was used. This is based on the base composition of cortical RNA reported by Mahler et al. (1966).

For analysis of lipids, about 150 mg of the frontoparietal cortex was dissected, weighed into 4.0 ml chloroform-methanol (1:1 v/v) and 0.4 ml water with 100 μg BHT (2,6-di-*tert*-butyl-*p*-cresol) as an antioxidant. After homogenisation, the homogenate was centrifuged at $2500 \times g$. The tissue residue was then extracted once more with 4.0 ml chloroform-methanol (3:1) and 0.1 ml water and filtered before the extracts were combined. Three milliliters of 1% NaCl in 0.01 M-HCl were added and, following centrifugation at $2500 g$ the upper layer was discarded. The lower layer was washed with methanol-water (1:1) and the chloroform phase evaporated with N_2 . The lipids were then dissolved in 5 ml of chloroform-methanol (2:1) and 15 μl , 0.5 ml, 3.0 ml, and 1.0 ml were taken for determinations of total phosphorus, total fatty acids (FA), free fatty acids (FFA), and individual phospholipids, respectively. The lipids were separated by tlc on silica gel 60. Light petroleum:diethyl ether:acetic acid (55:45:2, by vol.) and chloroform:methanol:acetic acid:water (C:M:HOAc:H₂O; 50:30:8:1, by vol.) were used as solvents for FFA and phospholipids, respectively. Phospholipids were eluted from the plates with C:M:HOAc:H₂O (50:39:1:10, by vol.) as described by Åkesson et al. (1970). Phospholipids were quantitated by phosphorus determination according to the method of Belfrage et al. (1970). The concentrations of individual phospholipids were calculated from the total lipid phosphorus and the percentage distribution of phosphorus between the different phospholipid classes. Total FAs were transesterified and FFAs (in the presence of silica gel) were esterified in 0.38 M-sulphuric acid in

TABLE 1. *Physiological parameters, cerebral blood flow, arteriovenous differences for oxygen, and cerebral metabolic rate for oxygen during hypoglycemia*

	Control ^a (n = 7)	Isoelectric EEG (min)	
		15 (n = 4)	30 (n = 4)
MABP (mm Hg)	149 ± 4	156 ± 6	134 ± 4
PaO ₂ (mm Hg)	108 ± 3	109 ± 6	111 ± 7
PaCO ₂ (mm Hg)	34.8 ± 0.8	33.7 ± 0.8	34.4 ± 1.5
pH	7.39 ± 0.01	7.38 ± 0.02	7.36 ± 0.04
CBF (ml·g ⁻¹ ·min ⁻¹)	1.10 ± 0.11	3.00 ± 0.30 ^b	2.54 ± 0.24 ^b
AVDO ₂ (μmol·g ⁻¹)	3.66 ± 0.22	1.34 ± 0.18 ^b	1.04 ± 0.28 ^b
CMRO ₂ (μmol·g ⁻¹ ·min ⁻¹)	3.95 ± 0.27	3.90 ± 0.30	2.65 ± 0.31 ^a

Results are means ± S.E.M.

MABP, Mean arterial blood pressure; CBF, Cerebral blood flow; AVDO₂, Arteriovenous differences for oxygen; CMRO₂, Cerebral metabolic rate for oxygen.

^a $P < 0.05$; ^b $P < 0.01$ (Mann-Whitney U-test).

^c Data from Sakabe and Siesjö (1979).

methanol:toluene (1:1 v/v) as described by Åkesson et al. (1970). The fatty acid methyl esters were separated by glc. Diethylene glycol succinate (10% on 80/100 Varaport 30) was used as the stationary phase in 2 m × 2 mm glass column with nitrogen as carrier gas. The temperature was programmed from 160° to 185°C at a rate of 1°C·min⁻¹.

Blood concentrations of β-hydroxybutyrate (β-HB) and acetoacetate (AcAc) were measured with a fluorometric modification of the spectrophotometric techniques described by Mellanby and Williamsson (1974)

and Williamsson and Mellanby (1974). Analytical conditions for measuring blood glucose concentrations have been previously described (Folbergrová et al., 1972b).

Statistics

Statistical differences were evaluated with Student's *t*-test when the material appeared to follow a normal distribution. In other cases the nonparametric Mann-Whitney U-test was applied.

RESULTS

Cerebral Oxygen Consumption

Table 1 shows the physiological data and the results of the CBF/CMRO₂ determinations in 2 groups of severely hypoglycemic animals, i.e., after 15 and 30 min, respectively, with an isoelectric EEG. The typical CBF response to hypoglycemia, a marked increase, was seen. The AVDO₂ was narrowed, and, as a result, the calculated CMRO₂ was maintained normal at 15 min and slightly decreased at 30 min. On the basis of these results and those reported in a previous communication (Norberg and Siesjö, 1976), we conclude that CMRO₂ is upheld at control levels in the slow wave polyspike period, and during the first 15 min of hypoglycemic 'coma.' The present results, demonstrating a reduction in CMRO₂ when coma is prolonged from 15 to 30 min, corroborate those previously obtained in man (Kety et al., 1948).

TABLE 2. *Blood glucose and arteriovenous difference for glucose compared with arteriovenous difference for oxygen at different levels of progressive hypoglycemia*

Group	EEG pattern	Blood glucose _{art} (μmol·g ⁻¹)	AVD _{gl} (μmol·g ⁻¹)	AVDO ₂ ^c (μmol·g ⁻¹)	AVD _{gl} × 6 AVDO ₂
1	Slow waves-polyspike (-60 min to -30 min) (n = 6)	2.00 ± 0.09	0.42 ± 0.04	4.36 ± 0.22	0.58 ± 0.03
2	Slow waves-polyspike (-30 min to 0 min) (n = 6)	1.20 ± 0.08	0.27 ± 0.03	3.40 ± 0.40	0.49 ± 0.03 ^a
3	"Iso" (0 min to 15 min) (n = 4)	0.59 ± 0.09	0.17 ± 0.03	3.37 ± 0.55	0.32 ± 0.05 ^{a,b}
4	"Iso" (30 min) (n = 5)	0.56 ± 0.10	0.16 ± 0.03	2.10 ± 0.33	0.48 ± 0.12

Values are means ± S.E.M. Statistical differences are only given for AVD_{gl} × 6/AVDO₂.

AVD_{gl}, Arteriovenous difference for glucose; AVDO₂, Arteriovenous difference for oxygen.

^a $P < 0.05$ group 2 and 3 compared with 1.

^b $P < 0.05$ group 3 compared with 2 (Mann-Whitney U-test).

^c The AVDO₂ was not decreased to the same extent as described in Table 1. In all probability this was due to the lower blood pressure and concomitant lower cerebral blood flow in the present experiments, in which the continuous venous outflow technique was used.

Cerebral Arterio-Venous Differences for Glucose and Oxygen

The results of glucose and oxygen determinations in paired or identical samples of arterial and cerebral venous blood are shown in Table 2. The samples are grouped according to the time of sampling with zero time defined as the time of onset of isoelectric EEG. To facilitate repeated sampling venous blood was obtained from the retrogenoid vein, which probably drains the whole brain (see Nilsson and Siesjö, 1976; 1980).

In the table, the left-hand column shows the decreasing glucose concentration in arterial blood during hypoglycemia. The right-hand column shows the quotient between 6 times the glucose uptake (shown in the second column) and the oxygen uptake (third column) during the same period. Usually in a normoglycemic brain where a complete oxidation of glucose accounts for almost all of the observed oxygen consumption, this quotient is expected to be close to 1. The greatly reduced value of this quotient during hypoglycemia demonstrates that considerable amounts of non-glucose substrates are oxidized by the brain in the slow-wave-polyspike period as well as in coma. The utilization of ketone bodies by brain during hypoglycemia can probably be ruled out. It has been shown that the uptake and utilization of ketone bodies by the brain is directly proportional to their levels in plasma, and that the utilization is negligible in the adult rat brain during control conditions (Hawkins et al., 1971; Sokoloff et al., 1977). Figure 1 shows that although the ketone body concentrations were increased in the starved rats not injected with insulin, hypoglycemia was accompanied by reductions in these concentrations. Under hypoglycemic conditions, blood concentrations of lactate and pyruvate are reduced as well (Lewis et al., 1974) and we failed to detect significant arteriovenous differences in lactate and pyruvate concentrations (data not shown).

From these results, we conclude that the missing substrates must be derived from endogenous metabolites. We will consider these substrates below, and discuss whether they can quantitatively account for the non-glucose oxygen consumption of the brain (see Discussion).

Phospholipid Degradation

As shown in Table 3, there was a 10% decrease in total fatty acid concentration after 5 min of isoelectric coma compared with control values. Prolongation of the isoelectric period to 60 min did not further decrease the total fatty acid pool. The ratio between the total fatty acid pool and total phospholipid phosphorus content was constant during all hypoglycemic periods, indicating that fatty acids from mono- and diglycerides and cholesterolesters

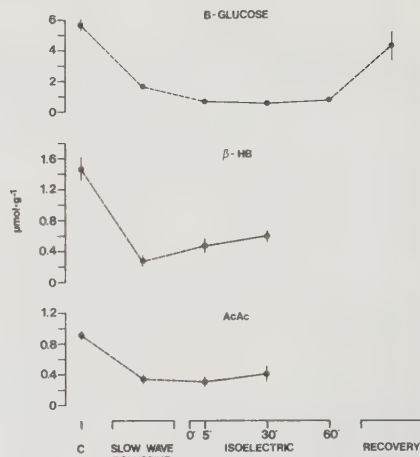


FIG. 1. Arterial blood glucose, β -hydroxybutyrate (β -HB), and acetoacetate (AcAc) concentrations in control (C) and insulin-injected animals with a slow wave-polyspike pattern and at different time intervals after onset of isoelectric EEG. The recovery values denote values obtained after 90 min of glucose infusion following an isoelectric period of 30 min. The glucose values (mean $\pm$ S.E.M.) represent pooled data from animals used for lipid analysis ($N = 6$). β -HB and AcAc were analysed from blood samples of the series used for AVD_{O_2}/AVD_{O_2} measurements ($N = 4-6$). All values are means $\pm$ S.E.M.

were not preferentially used as substrates. The total FFA concentration increased about sixfold after 5 min and this increase was sustained even after 60 min of isoelectric coma. However, this increase corresponds to only about 5% of the decrease of the total fatty acid pool.

In order to reveal if the decrease in the total fatty acid pool was accounted for by a preferential decrease in any single or group of fatty acids, the individual acids and phospholipids were analysed. In Table 4 the 5 major fatty acids are shown. There was a slight but sustained decrease in palmitic acid (16:0), stearic acid (18:0), and oleic acid (18:1) during hypoglycemic coma of 5 to 30 min duration. The most pronounced changes occurred in 18:1, which decreased about 16% or by $4 \mu\text{mol} \cdot \text{g}^{-1}$ cortex compared with control values. However, the decrease in 16:0 was only statistically significant in the 5-min group. There was no further decrease in the total concentrations of 16:0, 18:0, and 18:1 when the hypoglycemic coma was prolonged to 60 min. Arachidonic acid (20:4) decreased about 9% or by $1 \mu\text{mol} \cdot \text{g}^{-1}$ cortex in all hypoglycemic groups. No significant changes occurred in docosahexenoic acid (22:6).

Table 4 also shows the phosphorus content of the individual phospholipid classes: choline phospho-

TABLE 3. Total fatty acids, total phospholipid phosphorus concentrations, and total free fatty acids in rat cerebral cortex during hypoglycemia and in the recovery period following glucose administration

	Control	Isoelectric EEG (min)				Recovery 90 min following isoelectric EEG 30 min
		5	15	30	60	
Total fatty acids	119.1 ± 2.6	107.4 ± 2.8 ^a	108.4 ± 2.3 ^a	109.3 ± 1.9 ^a	110.0 ± 3.4	109.0 ± 2.3 ^a
Total phospholipid phosphorus	64.7 ± 1.5	59.5 ± 1.5 ^a	60.2 ± 1.0 ^a	60.1 ± 0.6 ^a	60.0 ± 0.9 ^a	58.7 ± 0.9 ^b
Total free fatty acids	0.12 ± 0.01	0.75 ± 0.07 ^c	0.74 ± 0.05 ^c	0.75 ± 0.08 ^c	0.96 ± 0.11 ^c	0.16 ± 0.04

The values are given in $\mu\text{mol} \cdot \text{g}^{-1}$ cortex wet weight and represent means $\pm$ S.E.M.

^a $P < 0.05$; ^b $P < 0.01$; ^c $P < 0.001$ (Student's *t*-test).

glycerides (CPG), inositol plus serine phosphoglycerides (IPG + SPG), and ethanolamine phosphoglycerides (EPG). During hypoglycemia, there was a similar decrease in both CPG and EPG, whereas SPG plus IPG were not measurably influenced.

As shown in Table 3, severe hypoglycemia is associated with a marked increase in the tissue concentrations of FFAs. Since such an increase had not been reported previously, we analyzed individual acids in the FFA pool. Figure 2 shows that the increase affected all FFAs analysed and that all values had returned to normal 90 min after glucose administration. The results of FFAs may have a bearing on the pathophysiology of hypoglycemic cell damage (see Discussion).

RNA Degradation

Electron microscopic findings reveal that there is a disintegration of ribosomes during the hypoglycemic period (see Introduction). The ribose unit

of the RNA molecule is a potential source of energy during glucose deprivation. Table 5 shows the total (mainly ribosomal) RNA content in the cerebral cortex of controls and in animals exposed to hypoglycemia with 30 min of isoelectric EEG. The control value of $1.50 \text{ mg RNA} \cdot \text{g}^{-1}$ cortex agrees well with the $1.25 \text{ mg RNA} \cdot \text{g}^{-1}$ rat cortex reported by Mahler et al. (1966) and with values tabulated by Rappoport et al. (1969). After 30 min of hypoglycemia the total RNA content is reduced by close to 10%, which is in agreement with the estimated ribosomal decrease previously reported. However, since the RNA content of brain is relatively low, the absolute decrease in RNA content only amounts to $0.41 \mu\text{mol} \cdot \text{g}^{-1}$ cortex. This corresponds to only 1% of the glucose equivalence value of the total fatty acid degradation.

DISCUSSION

As stated in the introductory section, severe hypoglycemia, especially if prolonged, is known to

TABLE 4. Major fatty acids and phospholipid phosphorus concentrations in rat cerebral cortex during hypoglycemia and in the recovery period following glucose administration

	Control	Isoelectric EEG (min)				Recovery 90 min following isoelectric EEG 30 min
		5	15	30	60	
Fatty acids						
Total ^a	119.1 ± 2.6	107.4 ± 2.8 ^c	108.4 ± 2.3 ^c	109.3 ± 1.9 ^c	110.0 ± 3.4	109.0 ± 2.3 ^c
16:0	28.5 ± 0.4	26.5 ± 0.6 ^c	26.6 ± 0.8	27.7 ± 0.7	27.0 ± 0.7	27.1 ± 0.7
18:0	26.2 ± 0.6	23.7 ± 0.5 ^c	24.1 ± 0.5 ^c	24.2 ± 0.5 ^c	24.4 ± 0.8	24.0 ± 0.5 ^c
18:1	25.5 ± 1.6	20.8 ± 0.9 ^c	21.3 ± 0.7 ^c	21.7 ± 0.7 ^c	21.9 ± 0.9	21.3 ± 0.7 ^c
20:4	11.7 ± 0.2	10.8 ± 0.2 ^c	10.8 ± 0.1 ^d	10.7 ± 0.2 ^d	10.5 ± 0.3 ^d	10.5 ± 0.2 ^d
22:6	19.1 ± 0.6	18.4 ± 0.6	17.8 ± 0.4	17.9 ± 0.3	18.2 ± 0.6	17.7 ± 0.7
Phospholipids						
Total ^b	64.7 ± 1.5	59.5 ± 1.5 ^c	60.2 ± 1.0 ^c	60.1 ± 0.6 ^c	60.0 ± 0.9 ^c	58.7 ± 0.9 ^d
CPG	25.6 ± 0.6	24.1 ± 0.5	23.7 ± 0.4 ^c	23.7 ± 0.4 ^c	23.6 ± 0.4 ^c	23.7 ± 0.6 ^c
IPG + SPG	9.5 ± 0.4	8.5 ± 0.3	8.9 ± 0.3	9.2 ± 0.3	8.7 ± 0.3	8.5 ± 0.2 ^c
EPG	26.4 ± 0.7	24.0 ± 0.7	24.7 ± 0.4 ^c	24.2 ± 0.2 ^d	24.3 ± 0.5 ^c	23.8 ± 0.4 ^d

The values are given in $\mu\text{mol} \cdot \text{g}^{-1}$ wet weight and represent means $\pm$ S.E.M.

^a Sum of fatty acids including 16:1, 18:2, 20:0, 20:1, 20:3, and 20:4.

^b Including lipid phosphorus in lysophosphatidylcholine ($0.2\text{--}0.4 \mu\text{mol} \cdot \text{g}^{-1}$) and sphingomyelin ($2.5\text{--}2.9 \mu\text{mol} \cdot \text{g}^{-1}$).

^c $P < 0.05$; ^d $P < 0.01$ (Student's *t*-test).

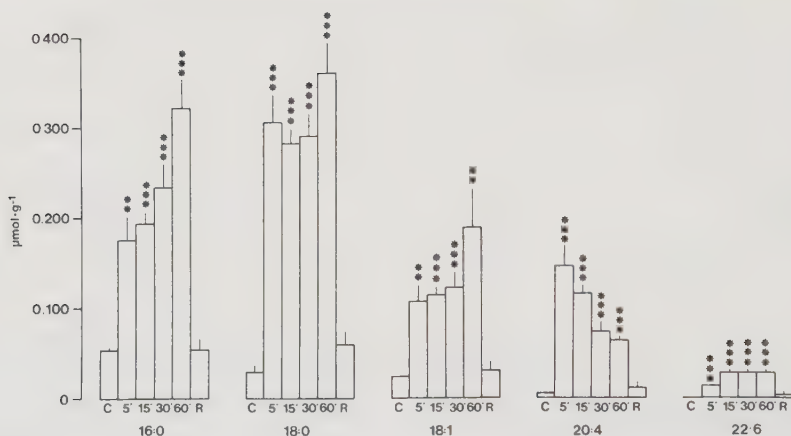


FIG. 2. Free fatty acid concentrations in cortical brain tissue (16:0, palmitic acid; 18:0, stearic acid; 18:1, oleic acid; 20:4, arachidonic acid; 22:6, docosahexenoic acid) in control (C) and experimental groups with an isoelectric EEG for 5 to 60 min and after 90 min of recovery following an isoelectric period of 30 min (R). Values are means $\pm$ S.E.M., $\mu\text{mol}\cdot\text{g}^{-1}$ wet tissue weight. **, ***. Significant increase in experimental group compared with control group ($P < 0.01$ and $P < 0.001$, respectively, Student's *t*-test).

cause irreversible neuronal damage in the brain. In most studies in which this has been established, whether clinical or experimental, it has been difficult to exclude an influence of arterial hypotension and/or hypoxemia as contributory causes. However, physiological parameters were carefully controlled in at least 2 experimental studies (Brierley et al., 1971; Agardh et al., 1980a; Kalimo et al., 1980) and, in these, unequivocal cell damage was demonstrated after periods of severe hypoglycemia of less than 60 min. During severe hypoglycemia, 2 major events occur that probably both contribute to the occurrence of the lesions. First, hypoglycemic coma (here defined as a degree of hypoglycemia that is accompanied by cessation of all spontaneous electrocortical activity) leads to extensive energy failure with marked decreases in the tissue concentrations of phosphocreatine and ATP, and increases in these of ADP and AMP (Hinzen and Müller, 1971; Lewis et al., 1974; Norberg and Siesjö, 1976). Second, the failing supply of exogenous glucose leads to consumption of endogenous substrate, some of which apparently emanates from structural

phospholipids (see introductory section). We will discuss in turn the nature of the endogenous substrates consumed, and the possibility that such consumption contributes to irreversible structural damage.

Oxidation of Endogenous Substrates

Previous results (Cravioto et al., 1951; Dawson, 1953; De Ropp and Snedeker, 1961; Tews et al., 1965; Goldberg et al., 1966; Lewis et al., 1974; Feise et al., 1976; Norberg and Siesjö, 1976) showed that severe hypoglycemia gives rise to utilisation of endogenous substrates such as glycogen, glucose, glycolytic metabolites, citric acid cycle intermediates, and free amino acids. As discussed in the introductory section, severe hypoglycemia in intact animals also seems to affect the tissue concentrations of proteins or nucleic acids, but to a lesser extent than that of phospholipids.

In the present study, we attempted to evaluate the nature of the endogenous substrate utilised in hypoglycemic coma. This attempt was facilitated by previous studies in which changes in carbohydrate and amino acids were followed as a function of time of hypoglycemia (Lewis et al., 1974; Norberg and Siesjö, 1976; Agardh et al., 1978). Based on the observations, sequential changes in blood and brain concentrations of metabolites and macromolecules during the progression from control conditions to the slow wave/polyspike period to the isoelectric coma period, we can estimate the 'glucose equivalence value' of these changes (assuming complete

TABLE 5. Total RNA content in rat cerebral cortex following 30 min of hypoglycemic coma

	mg·g ⁻¹	$\mu\text{mol}\cdot\text{g}^{-1}$
Control	1.50 $\pm$ 0.02	4.41 $\pm$ 0.06
Iso 30 min	1.36 $\pm$ 0.01 ^a	4.00 $\pm$ 0.03 ^a

The values are means $\pm$ S.E.M.

^a $P < 0.001$; Student's *t*-test.

oxidation of the compound in question), and thereby evaluate which substrate utilisation accounts for the 'O₂ deficit' at the different stages. For the purpose of comparison, we divided hypoglycemia into 3 progressive stages:

Stage 1, 'Control to slow wave,' covers the blood glucose range from normal to $2 \mu\text{mol} \cdot \text{g}^{-1}$. Stage 2 is 'Slow wave to isoelectricity of 5–15 min duration'. Stage 3 is 'Isoelectricity of 15 to 30 min duration'.

Stage 1

By the time the blood glucose value has been reduced to around $2 \mu\text{mol} \cdot \text{g}^{-1}$, there has been a progressive reduction in many of the brain metabolites (Lewis et al., 1974; Norberg and Siesjö, 1976). Brain glucose is depleted, brain glycogen is approximately 60% reduced, and there have been decreases in the levels of glycolytic and citric acid cycle intermediates and amino acids, notably those of glutamine and glutamate, even though these changes are partly offset by an increase in the aspartate level. Our (*unpublished*) results indicate that there is no change in total fatty acid concentration in this stage.

By adding up these concentration changes, one finds that they correspond to a total of about $6 \mu\text{mol} \cdot \text{g}^{-1}$ glucose equivalents. This means that oxidation of endogenous metabolites can account for the required O₂ utilisation for approximately 20 min when the O₂ deficit corresponds to $0.3 \mu\text{mol} \cdot \text{g}^{-1} \cdot \text{min}^{-1}$ of glucose equivalents (see Tables 1 and 2). The duration of the slow wave-polyspike phase differs very much from one rat to another, and may last between 30 and 60 min. It is conceivable that, in this period, the missing substrate is made up of the endogenous carbohydrate and amino acid compounds considered.

Stage 2

During the transition from the slow wave-polyspike phase through the first 15 min of isoelectric coma, the utilisation of endogenous metabolites continues. Brain glycogen is depleted during this phase, and all the glycolytic and citric acid cycle intermediates and associated amino acids attain their minimum levels, with the exception of aspartate and succinate, whose levels increase. The combined metabolite changes converted into glucose equivalents amount to a total of $2.6 \mu\text{mol} \cdot \text{g}^{-1}$, or enough to sustain the required O₂ utilisation for 10 min. However, during this stage there is also a massive decrease in the total brain fatty acids and P-lipid phosphorus as discussed below.

Stage 3

There are no further changes in soluble metabolites during stage 3, and the required O₂ uptake has

to be accounted for by the oxidation of macromolecules such as fatty acids and RNA.

As a first approximation, one may conclude that the majority of the substrates considered are consumed during the first 5 min of coma, leaving an unexplained non-glucose oxygen consumption of about $2.0 \mu\text{mol} \cdot \text{g}^{-1} \cdot \text{min}^{-1}$ for the period 5–30 min (see Results). During this period, other substrates therefore must have provided $0.33 \mu\text{mol} \cdot \text{g}^{-1} \cdot \text{min}^{-1}$ of glucose equivalents.

Our results, demonstrating an 8% reduction in the phospholipid content of the cerebral cortex during hypoglycemic coma, corroborate those previously reported by others (see introductory section), although the results from Hinzen et al. (1970) gave a surprisingly large reduction in the phospholipid content of the rabbit brain. The complete oxidation of $10 \mu\text{mol}$ of fatty acids of the chain length observed in this study, plus the oxidation of the $5 \mu\text{mol}$ of the glycerol moiety corresponds to $39 \mu\text{mol} \cdot \text{g}^{-1}$ glucose equivalents. Therefore, fatty acid oxidation would obviously cover more than all the non-glucose oxygen consumption during hypoglycemic coma. However, two problems arise. First, the possibility must be considered that disappearance of phospholipids represents peroxidative degradation rather than oxidation. Second, it is necessary to take into account the fact that the phospholipid pool is reduced during the first 5 min of hypoglycemic coma with no further change thereafter.

Oxidation Versus Peroxidation

When studied *in vitro* in the presence of suitable free radical initiators brain tissues have a large peroxidative capacity (Barber and Bernheim, 1967; see also Rehnrcrona et al., 1980). However, peroxidative changes in lipids preferentially affects the polyenoic fatty acids (20:4 and 22:6) and ethanolamine phosphoglycerides (Rehnrcrona et al., 1980). No such preferential loss of polyenoic fatty acids were observed in the present material, and we tentatively conclude that any peroxidative degradation of tissue lipids was small.

Phospholipid Degradation and Free Fatty Acid Increase

The increase in FFAs observed presently is similar to that previously noted in ischemia both in terms of magnitude of accumulation and percentage distribution of fatty acids (Bazán, 1970; 1976; Cenadella et al., 1975; Marion and Wolfe, 1979; Rehnrcrona et al., 1981). The accumulation during hypoglycemia is in accordance with the hypothesis that activation of phospholipases occurs in conditions in which ATP concentrations fall, and Ca²⁺ is released from intracellular binding sites (Markelonis

and Garbus, 1975; see also Flower and Blackwell, 1976).

If the purpose of the hydrolysis of phospholipids and the release of FFAs was to mobilize endogenous storage materials for substrate utilisation under these conditions, then it is difficult to explain why the decrease in tissue phospholipids was limited to the first 5 min of hypoglycemic coma, and why this initial degradation ($11.7 \mu\text{mol} \cdot \text{g}^{-1}$) was 25 to 30 times in excess of what could be processed by normal fatty acid oxidation based on the observed amount of cerebral oxygen consumption. One could postulate the conversion of the phospholipid degradation products to partially oxidized 'intermediates,' which could be utilised gradually during the remaining coma period. However, an accumulation of short and intermediate length fatty acids (our analysis system does not detect fatty acids shorter than C:16) is contrary to the known mechanism of β -oxidation of fatty acids and is therefore improbable. It must therefore be suspected that there was a significant transport of free fatty acids (which would be toxic to the tissue in high amounts) from the tissue to the blood during the initial coma period.

Summarising the data on changes in potential substrates during hypoglycemia we arrive at the following conclusions. First, it appears probable that endogenous carbohydrate metabolites and free amino acids provide most of the missing substrate in the slow wave-polyspike period. Second, although further changes in these substrates occur in 'coma,' these cannot quantitatively account for the non-glucose oxygen consumption in the 0–30 min period. Third, although breakdown of phospholipids during the first 5 min of isoelectricity may provide the missing substrate, interpretation is hampered by the facts that no further breakdown occurred thereafter, that the increase in the FFA pool only corresponded to about 5% of the reduction in total fatty acids, and that the FFA pool remained constant during 60 min of isoelectricity. It remains to be shown if and how fatty acids are utilised by the brain in hypoglycemia, and it cannot be excluded that unmeasured substrates were oxidized.

Relationship to Structural Damage

In contrast to the previous study, which dealt with a much shorter hypoglycemic period in rats (until righting reflex was lost; Samson et al., 1959), the present one clearly showed that severe hypoglycemia was accompanied by a significant reduction in the tissue RNA concentration. The amount disappearing was too small to be significant in terms of substrate provision. However, the results offer an interesting parallel to the electron microscopic alterations observed under similar conditions of hypoglycemia (Kalimo et al., 1980). In that study,

hypoglycemic coma of 30 (and 60) min duration was accompanied by loss of endoplasmic reticulum in neurons, and by disappearance of ribosomes. Probably, this loss corresponds to the decrease in RNA observed presently.

At present, the source of the phospholipids lost during hypoglycemia is not known but they could be derived from intracellular membranes in mitochondria and other subcellular structures. Clearly, a partial degradation of such membranes must have serious consequences for cell function. For example, a deficient membrane function of mitochondria could well account for the fact that cerebral energy state is deranged in spite of an upheld cerebral oxygen consumption. Free fatty acids are known to interfere with several aspects of mitochondrial function (Wojtczak, 1976). It is therefore possible that the accumulation of FFA may disturb the mitochondrial function. Another potentially adverse factor is the accumulation of arachidonic acid that occurs during hypoglycemia (see Fig. 2). Thus, this accumulation may trigger an increased synthesis of prostaglandins and related substances, i.e., of compounds that have been implicated in causing cell damage in other tissues (see Flower, 1979). In the future, work must be directed toward those subcellular events that underly irreversible cell damage in hypoglycemia.

ACKNOWLEDGMENTS

The skillful assistance of Kerstin Beirup, Lena Sjöberg, Gertie Johansson, Karin Hansson, Barbro Asplund, and Yvonne Hansson is gratefully acknowledged. We are also grateful for helpful discussions with Dr. Björn Åkesson and Eva Westerberg, B.Sc.

This study was supported by grants from the Swedish Medical Research Council (No. 14X-263), from U.S. Public Health Service (No. R01 NS07838), from the Knut and Alice Wallenberg Foundation, and from Thorsten and Elsa Segerfalk Foundation.

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Hypoglycemic Brain Injury

I. Metabolic and Light Microscopic Findings in Rat Cerebral Cortex During Profound Insulin-induced Hypoglycemia and in the Recovery Period Following Glucose Administration

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Summary. Profound hypoglycemia causing the disappearance of spontaneous EEG activity was induced by insulin in rats. For analysis of cerebral cortical concentrations of labile phosphates, glycolytic metabolites and amino acids, the brain was frozen in situ. For microscopic analysis of the corresponding cerebral cortical areas the brain was fixed by perfusion. Hypoglycemia with an isoelectric EEG for 30 and 60 min caused severe perturbation of the cerebral energy metabolites. After both 30 and 60 min of isoelectric EEG, two microscopically different types of nerve cell injury were seen. Type I injury was characterized by angulated, darkly stained neurons with perineuronal vacuolation, mainly affecting small neurons in cortical layer 3. Type II injured neurons, mainly larger ones in layers 5–6, were slightly swollen with vacuolation or clearing (depending on the histotechnique used) of the peripheral cytoplasm, but had no nuclear changes.

Recovery was induced by glucose injection. Improvement in the cerebral energy state occurred during the 30 min recovery period even after 60 min of hypoglycemia. However, the persisting reduction in the size of adenine nucleotide and amino acid pools after 30 or 180 min recovery suggested that some cells remained damaged. In confirmation many type I injured neurons persisted during the recovery suggesting an irreversible injury. The disappearance of virtually all type II injuries indicated reversibility of these histopathological changes.

The microscopic changes in hypoglycemia were different from those in anoxia-ischemia suggesting a dissimilar pathogenesis in these states despite the common final pathway of energy failure.

Key words: Hypoglycemia — Nerve cell injury — Biochemistry — Light microscopy — Rat cerebral cortex

In hypoglycemia the brain is deprived of glucose, its principal substrate for energy production. When hypoglycemia is of sufficient severity it leads to gross energy failure causing functional derangement, such as abolition of electroencephalographic (EEG) potentials and clinical coma (Tews et al. 1965; Hinzen and Müller 1971; Lewis et al. 1974b; Norberg and Siesjö 1976; Agardh et al. 1978). If it is of sufficient duration it causes irreversible neuronal injury (Brierley et al. 1971a).

Energy failure also occurs in anoxia-ischemia. The light microscopic neuronal changes in hypoglycemia, as well as the pathogenesis of the nerve cell injury, have been considered identical to those in anoxia-ischemia (Brierley et al. 1971b; Brierley 1976). In some cases there are, however, certain differences in the distribution of the brain lesions in hypoglycemia as compared to ischemia both in human neuropathological cases (Kalimo and Olsson, in prep.) and in some experimental conditions (Myers and Kahn 1971). Furthermore from the biochemical point of view the metabolic events, which in hypoglycemia precede the appearance of gross energy failure, are markedly different from those in anoxia-ischemia. In hypoglycemia the exhaustion of the carbohydrate stores, and the utilization of non-carbohydrate substrates for energy production with ample oxygen present, terminally result in decreased levels of lactate, pyruvate, citric acid cycle metabolites, NADH and certain amino acids, whereas the concentration of ammonia increases excessively as a consequence of oxidative deamination. Furthermore, as a result of the substrate depletion, the intracellular pH is not decreased (Lewis et al. 1974a; Pelligrino et al. 1979). This contrasts with anoxia-ischemia, where the lactate concentration and lactate/pyruvate ratio increase with a concomitant fall in pH and NAD^+ . In addition, the rise in ammonia level is less pronounced than in hypoglycemia. Thus, these results demonstrate that despite a common final event leading to depletion of energy-rich compounds there are important patho-

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Table 2. Physiological parameters during insulin-induced hypoglycemia and in the recovery period following glucose administration

Experimental group	Isoelectric EEG (min)	Recovery (min)	No. of animals	MABP (mm Hg)	P_{aO_2} (mm Hg)	P_{aCO_2} (mm Hg)	pH	Temp. (°C)
A (Controls)			23	152 ± 4	112 ± 3	37.1 ± 1.0	7.343 ± 0.011	37.1 ± 0.1
B	30	—	9	153 ± 10	124 ± 4	36.2 ± 2.0	7.317 ± 0.021	37.0 ± 0.1
C	60	—	12	119 ± 11	104 ± 5	35.9 ± 1.2	7.295 ± 0.016	37.0 ± 0.1
D	30	30	9	149 ± 7	120 ± 8	35.4 ± 1.1	7.254 ± 0.021	37.0 ± 0.1
E	30	180	7	126 ± 8	106 ± 7	37.2 ± 1.2	7.240 ± 0.031	37.3 ± 0.1
F	60	30	10	147 ± 5	111 ± 5	33.2 ± 0.9	7.254 ± 0.023	37.2 ± 0.2

The values are means ± S.E.M. MABP = mean arterial blood pressure

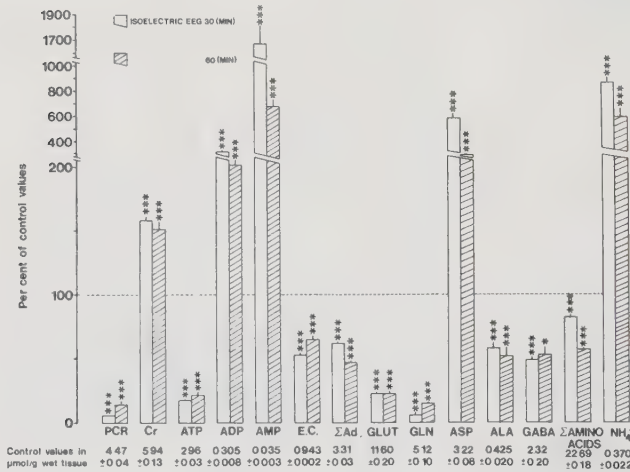


Fig. 3. Cerebral cortical concentrations of PCr, Cr, ATP, ADP, AMP, glutamate, glutamine, aspartate, alanine, GABA, ammonia (NH_4^+) and energy charge (E.C.), sum of adenine nucleotides (ΣAd) and sum of amino acids during hypoglycemia with an isoelectric EEG for 30 and 60 min. The values are means ± S.E.M. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

metabolic and histopathologic changes observed. In animals with 60 min of severe hypoglycemia, and in the recovery groups, arterial pH was moderately reduced.

In all animals the brains were firmly fixed and no blood remained in the blood vessels. The morphology of the control animals was such as generally considered normal. This was further verified at the light microscopic high-resolution level using semithin sections of epon-embedded material in which no injured neurons (dark or vacuolated) were encountered. In this treatise emphasis is laid on neuronal changes in the cerebral cortical areas corresponding to the biochemical analyses. A more detailed topographical study on the hypoglycemic brain injury is the subject of a separate communication.

Events Occurring During Hypoglycemia

Biochemical Changes. A previous study (Agardh et al. 1978) concerned cortical metabolic changes in severe

hypoglycemia of maximally 30 min duration. In the present series we evaluated metabolic changes after 60 min of ceased EEG activity. Figure 3 shows changes in organic phosphates and amino acids in the 30 and 60 min groups. Changes in glycolytic metabolites (not shown) after 60 min were similar to those previously reported after 30 min. Thus, glycogen and glucose were virtually depleted and concentrations of pyruvate and lactate markedly reduced. The only difference was that in the present material (60 min), the lactate/pyruvate ratio was not reduced below control values (the values were 15.6 ± 1.0 and 16.3 ± 2.4 , respectively). However, since the ratio was not increased we conclude that cellular hypoxia did not contribute to the histopathologic alterations observed.

As Fig. 3 shows, prolongation of the period of hypoglycemia to 60 min did not further alter PCr, creatine, or ATP concentrations, nor did it cause any additional reduction in the adenylate energy charge (E.C.). However, ADP and AMP concentrations were



Fig. 4. Cerebral cortical layer 3 from a rat exposed to 30 min of hypoglycemia with isoelectric EEG. Both condensed, angular neurons with perineuronal vacuoles (type I injury, *arrowheads*) and a swollen neuron with subplasmalemmal vacuoles (type II injury, *thick arrow*) are seen. Besides neurons with slightly condensed cytoplasm (a likely incipient type I injury) and with more or less normal appearance are found next to the injured ones. Note a dark dendrite of a type I neuron traversing the neuropil (*thin arrow*). JB-4 embedding and toluidine blue staining. $\times 460$

Fig. 5. Thirty minutes of hypoglycemia, cortical layer 3 from Epon-embedded material. The vacuoles in connection with the condensed type I injured neurons are extracytoplasmic (*arrows*). The type II injured neuron (*thick arrow*) displays peripheral clearing of the cytoplasm. The neuropil is somewhat spongy. Epon and toluidine blue. $\times 460$

Fig. 6. Thirty minutes of hypoglycemia, cortical layer 3. A swollen type II injured neuron in which the subplasmalemmal vacuoles extend into the apical dendrite. JB-4 and toluidine blue. $\times 720$

Fig. 7. Sixty minutes of hypoglycemia, cortical layer 3. Both the number of affected neurons and the severity of the type I and II injury are somewhat increased as compared to Fig. 4. JB-4 and toluidine blue. $\times 460$

Fig. 8. Sixty minutes of hypoglycemia, cortical layer 3. A type I (*arrowhead*) and a type II (*thick arrow*) injured neuron next to relatively normal appearing neurons. Note the condensed dendrite of the type I injured neuron and the membrane whorls in the subplasmalemmal clear zone of the type II injured neuron (*thin arrow*). The nuclear chromatin in the type II injured neuron is evenly distributed. Epon and toluidine blue. $\times 760$

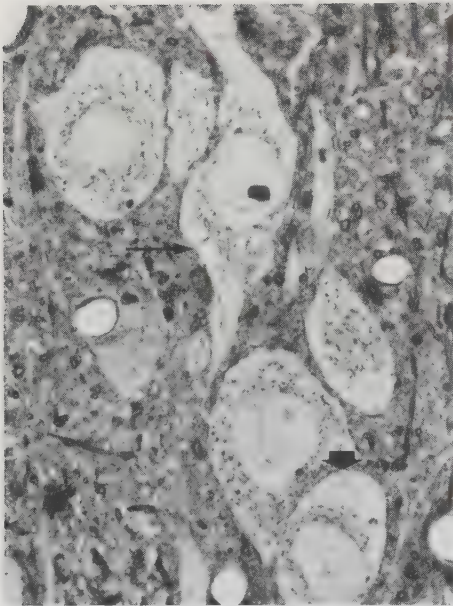


Fig. 9. Sixty minutes of hypoglycemia, cortical layer 5–6. All the type II injured neurons show the peripheral clear zone of cytoplasm, and in one of them subplasmalemmal vacuoles are seen even in Epon-embedded material (*thin arrow*). In another neuron there are whorls of membranes in the clear zone (*thick arrow*). Nuclear chromatin is evenly dispersed. Epon and toluidine blue. $\times 1,150$

Table 3. Relative proportions of normal appearing as well as of type I and type II injured neurons in cerebral cortex of rats exposed to hypoglycemia with and without recovery

Group	Normal appearing	Type I	Type II
B (30 min)	80.0%	13.9%	6.1%
C (60 min)	59.1%	31.1%	9.8%
D (30 min + 30 min)	92.4%	7.4%	0.2%
E (30 min + 180 min)	98.0%	1.8%	0.2%
F (60 min + 30 min)	77.8%	22.0%	0.2%

reduced. As a result, the size of the adenine nucleotide pool decreased to below 50% of control.

Prolongation of the severe hypoglycemia to 60 min did not further exaggerate the changes observed after 30 min in the concentrations of glutamate, glutamine, alanine or GABA. There was a marked fall in aspartate concentration, though, with a resulting reduction in the size of the amino acid pool (from 18.04 ± 0.46 to $12.87 \pm 0.20 \mu\text{mol} \cdot \text{g}^{-1}$).

Histopathologic Alterations. The neuronal cell injury characteristically displayed two different patterns: In type I injury (Fig. 4) the neurons showed (with all three histological techniques used, vide *infra*) marked shrinkage with triangular shape and condensation of both their nuclei and cytoplasm. The plasma membrane was scalloped and small perineuronal vacuoles were common. These vacuoles seemed to be virtually always extracellular (Fig. 5) as opposed to the intracytoplasmic vacuoles described in certain types of anoxia-ischemia. The processes of these condensed neurons were often visible as dark, tortuous bands traversing the neuropil (Figs. 4 and 8). Some neurons with essentially normal size and form and without perineuronal vacuolization showed slightly increased intensity of the cyto- and karyoplasmic staining, possibly indicating an incipient type I injury (Fig. 4).

In the type II injury the neurons appeared slightly swollen. In these neurons the characteristic feature was the empty looking vacuoles underneath the plasma membrane as seen in JB-4 and paraffin embedded sections (Figs. 4, 6, 7). Vacuolization sometimes extended into the dendrites of these neurons (Fig. 6). In semithin Epon-sections, however, no vacuoles were usually seen, but only subplasmalemmal clear zones (Figs. 8 and 9). The nucleus appeared normal with finely dispersed chromatin and prominent nucleoli (Figs. 4–9). No perineuronal extracytoplasmic vacuolization was seen around the neurons with type II injury. As a possible change preceding the subplasmalemmal vacuolization, the cytoplasm of some neurons appeared reticulated.

Qualitatively, 30 min or 60 min hypoglycemia caused essentially similar nerve cell injury, but quantitative differences were seen with regard to the number of affected neurons (Table 3) and the severity of the changes in individual neurons. On the average the type I injured neurons showed more intense condensation and perineuronal vacuolization and in type II injured neurons the cytoplasmic vacuolization/clearing was more extensive after 60 min hypoglycemia (Fig. 7). Furthermore, after 60 min subplasmalemmal vacuoles were seen in a few neurons even in Epon-embedded material, and whorls of refractile membranous material were accumulating in the clear zones of type II injured neurons (Figs. 8 and 9). The nuclei of these neurons had still evenly dispersed chromatin and prominent nucleoli.

Remarkably, no transition between these two types of nerve cell injury was seen. The majority of the cortical neurons not showing either type I or II injury were slightly rounded in shape with dispersed Nissl substance. The distribution of the injured neurons in the cortex was uneven. The small neurons, especially those in layer 3, were the most vulnerable ones, while

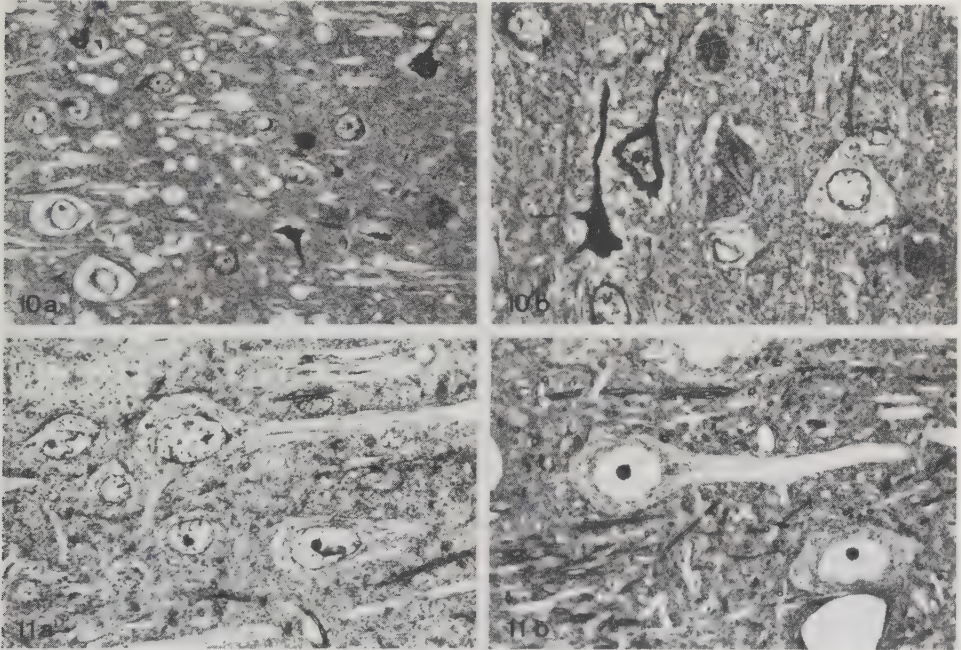


Fig. 10a and b. Sixty minutes of hypoglycemia, cortical layer 3. In FAM-fixed and JB-4-embedded material the type I injured neurons are fairly similar as in GA-fixed material. In type II injured neurons the cytoplasm appears "washed-out" and the nuclei are empty-looking. JB-4 and toluidine blue. **a:** $\times 400$, **b:** $\times 790$

Fig. 11a and b. The difference between the FAM (**a**) and GA (**b**) becomes evident in the Epon-embedded control material. In FAM-fixed tissue the nuclear chromatin is clumped, cellular outlines fuzzy, and the cellular processes in the neuropil less distinct than in the GA-fixed tissue. Epon and toluidine blue. $\times 720$

the neurons of layers 5–6 and larger neurons in general were less often affected. It appeared that the small neurons more often showed type I lesion whereas type II injury was more common in larger neurons. This conclusion is, however, hampered by the fact that type I injury causes shrinkage and type II slight swelling of the neurons.

Our cell counts showed that the greatest number of affected neurons was present over the lateral aspects of the cortex, most often definitely lateral to the arterial border zone between the anterior (ACA) and middle (MCA) cerebral arteries. Notably the cortex at the vertex and on the medial surface (i.e. the ACA supply area) was affected to a lesser extent. In the areas of most severe involvement there was sponginess of the neuropil and swelling of perivascular astrocytic processes.

The morphology of FAM-fixed cortex in control rats and those exposed to 60 min of hypoglycemia was different from that in GA-fixed rats. In general, the cellular outlines appeared somewhat fuzzier and the

nuclei were rarefied, often empty-looking (Figs. 10 and 11). The cytoplasm of type II injured neurons was more severely vacuolated, often entirely "washed-out" (Fig. 10a and b). However, the outlook of condensed type I injured neurons was approximately the same as in GA-fixed tissue.

Events During Recovery

Biochemical Changes. Figures 12 and 13 demonstrate cerebral metabolite concentrations 30 min after glucose administration following isoelectric periods of 30 min (Agardh et al. 1978) and 60 min (present data). The results suggest that prolongation of the isoelectric period to 60 min aggravated the posthypoglycemic metabolic perturbation. After 30 min of recovery following 60 min of hypoglycemia, blood glucose concentrations rose to a mean value of $7.94 \pm 0.8 \mu\text{mol g}^{-1}$ compared to 4.10 ± 0.66 in the group with 30 min of recovery following 30 min of hypoglycemia. This

probably explains why tissue glucose concentrations increased (Fig. 12). After 60 min of hypoglycemia both the lactate and pyruvate concentrations rose but since the lactate/pyruvate ratio did not increase the results failed to indicate that gross mitochondrial damage had occurred (see Discussion).

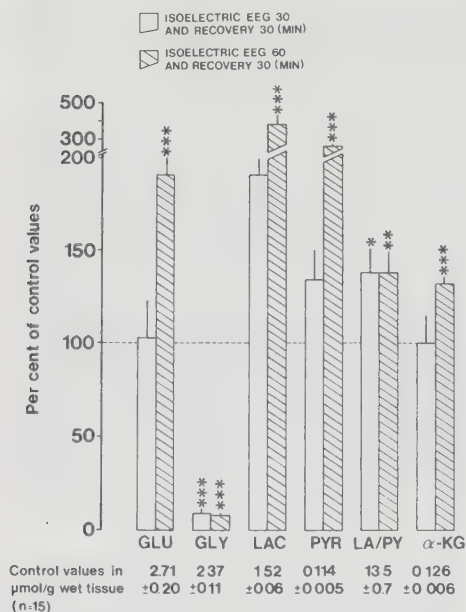


Fig. 12. Cerebral cortical concentrations of glucose, glycogen, lactate, pyruvate, α -ketoglutarate, and the lactate-pyruvate ratio (La/Py) after 30 and 60 min of isoelectric EEG followed by 30 min of recovery. The values are means $\pm$ S.E.M. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

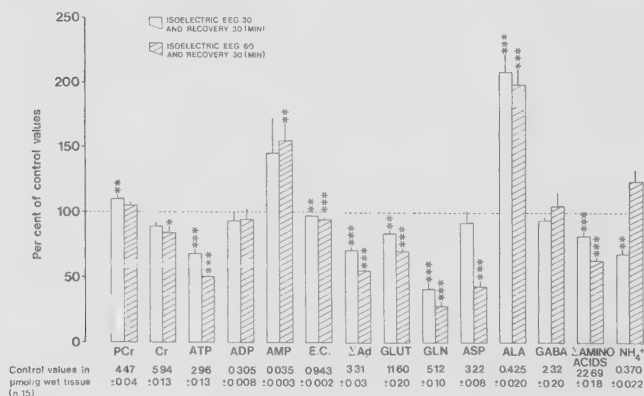


Fig. 13. Cerebral cortical concentrations of PCr, Cr, ATP, ADP, AMP, glutamate, glutamine, aspartate, alanine, GABA, ammonia (NH_4^+) and energy charge (E.C.), sum of adenine nucleotides (Σ Ad) and sum of amino acids after 30 and 60 min of isoelectric EEG followed by 30 min of recovery. The values are means $\pm$ S.E.M. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

As Fig. 13 shows, there was extensive recovery of cerebral energy state also after 60 min of hypoglycemia, and the changes observed were dominated by a persisting reduction in the size of the adenine nucleotide pool (Σ Ad). This could at least partly have been due to insufficient time for resynthesis since the amount reformed ($0.25 \mu\text{mol} \cdot \text{g}^{-1}$) was not grossly different from that observed in the 30 min isoelectric group ($0.31 \mu\text{mol} \cdot \text{g}^{-1}$). However, some of the changes observed suggest the presence of metabolic damage. Thus, although the PCr and ADP concentrations were close to control, there was a significant increase in the AMP concentration, and a larger decrease in the energy charge than after 30 min of hypoglycemia.

Changes in amino acids (Fig. 13) were dominated by marked reduction in concentrations of glutamate, aspartate, and glutamine and by accumulation of alanine. Only the concentrations of GABA and ammonia returned to control values. There was little resynthesis of amino acids and in the 60 min group the size of the amino acid pool was still reduced by about $8 \mu\text{mol} \cdot \text{g}^{-1}$. After 180 min of recovery following 30 min of isoelectric EEG there was extensive resynthesis of most metabolites. However, the ATP concentration was still reduced and the amino acid pool significantly decreased ($P < 0.001$) (Agardh et al. 1978).

Histopathologic Alterations. During the recovery neurons with type II injury had practically disappeared (Table 3). Some cells stained slightly lighter, possibly representing recovered type II neurons. Neurons with type I injury had similar appearance and distribution as in animals without restitution. Their number decreased during recovery but even after 180 min of restitution following 30 min of hypoglycemia they still constituted approximately 2% of the neurons (Table 3). Besides, Nissl substance in many neurons still appeared less compact than in normal animals.

Discussion

Clinical and neuropathological experience has shown that hypoglycemia of sufficient severity may lead to permanent coma and extensive necrotic changes in the brain. The pathogenesis of such tissue changes is complex and still poorly understood. Primary cellular changes and reactive alterations in glial cells and nutrient blood vessels may all be involved in the pathogenesis of the permanent injuries. In human cases dying of hypoglycemic brain injury usually only the end result of the injury can be examined. Furthermore, there are remarkably few experimental studies in which morphology has been correlated with functional and biochemical findings (Kahn and Myers 1971; Myers and Kahn 1971; Meldrum et al. 1971; Brierley et al. 1971b) and in none of them electron microscopy has been utilized.

We have started a series of such correlative experimental investigations on hypoglycemic brain injury. Our experimental model relies on grading the severity of the hypoglycemic insult on the basis of the duration of the EEG silence during which biochemical parameters relevant to the derangement of the cellular energy metabolism are measured. The morphological results are correlated with biochemical parameters recorded from similar experiments with corresponding insults.

When glucose is administered after a 30 min period of severe hypoglycemia, associated with gross energy failure, there is relatively extensive recovery of cerebral energy state (Agardh et al. 1978). However, since increases in lactate concentrations and in the lactate/pyruvate ratio were observed, and since there was a persisting reduction in the size of the adenine nucleotide pool, we concluded that some cell damage was probably present. The present results provide direct proof that such damage is incurred.

Although the present results suggest that prolongation of the hypoglycemic period to 60 min aggravates the perturbation of cerebral metabolism, changes observed during the hypoglycemia were dominated by further reductions in the sizes of the adenine nucleotide and amino acid pools. In all probability, the reduction in adenine nucleotide pool size was due to continued dephosphorylation and deamination of AMP to purine nucleosides and bases (Deuticke et al. 1966; Kleihues et al. 1974; Siesjö 1978). Since some of these degradation products are diffusible they can leave the tissue via the circulation. Since only the remaining nucleosides and bases can be used for (rapid) re-synthesis by salvage pathways, and since *de novo* synthesis is slow, this loss partly explains the persisting reduction in pool size. The further reduction in amino acid pool size in the 60 min group probably reflects mobilization of amino acid carbon for oxidation via the

glutamate dehydrogenase reaction. Since there was no associated rise in ammonia concentration the results suggest that ammonia left the tissue via the circulation. In the 30 min recovery period, the grossly perturbed glutamate/aspartate ratio returned towards control values and GABA concentrations normalized. However, little resynthesis of amino acids were observed and alanine was markedly elevated. Although these changes hint an abnormal amino acid metabolism experiments with long recovery periods are required to show whether or not the changes observed represent irreversible events.

After 30 and, especially, after 60 min of ceased EEG activity there is poor recovery of brain function (Agardh and Rosén, *in prep.*) and the present results indicate that many cells are irreversibly damaged. In view of this, it is somewhat surprising that PCr concentrations returned to normal and that the adenylate energy charge was only moderately reduced. There are two possible explanations. First, it is conceivable that if the recovery period is prolonged, following 60 min of severe hypoglycemia, the number of irreversibly damaged cells may turn out to be smaller than indicated by the figures at 30 min of recovery. In other words, it cannot be excluded that some cells with type I injury retain some basic metabolic functions, including oxidative phosphorylation. Second, since neurons occupy only part of the total cell population it is clear that alteration reflecting metabolic injury to neurons are considerably attenuated when the total cell mass is analysed.

As expected, neurons displayed the most severe morphological changes, the distribution and form of which varied from one region to another. Two distinctly different types of nerve cell alterations were encountered. Neither of them exactly corresponds to those changes previously described in anoxia-ischemia, with which the hypoglycemic injury has been considered identical (Brierley 1976). The condensed, angulated neurons of the hypoglycemic type I injury do not show the cytoplasmic microvacuolation which is characteristic of incomplete and/or transient anoxia-ischemia (Brown and Brierley 1972; Garcia et al. 1973, 1977; Arsenio-Nunes et al. 1973), though in other respects these cell changes resemble each other. It must also be born in mind that dark neurons always call for caution in interpretation, since such neurons are easily produced as artifacts in improperly fixed tissue (Cammermeyer 1972). As we do not see dark neurons in our control animals fixed and processed for microscopy identically as in our experiments with hypoglycemia their presence must indicate at least altered reactivity towards the processing or, as we feel more likely, a nerve cell injury already present *in vivo* at the moment of fixation. Furthermore, such cells are more

abundant in rats with 60 min of hypoglycemia than in the 30 min group, and their number decreases in animals with recovery. These are additional facts corroborating our view that type I alteration represents an *in vivo* injury.

The neurons with type II injury has a characteristic appearance not resembling any type of anoxic-ischemic cell change, nor any other established form of nerve cell injury which we are aware of. It should be pointed out that the subplasmalemmal vacuolization is dependent on the choice of embedding medium, since in Epon-sections most often only clearing of the peripheral cytoplasm due to changes in the ribosome-coated endoplasmic reticulum can be seen (Kalimo et al. 1980). This underlines the importance of taking into consideration the morphological methods used in the interpretation of the cellular changes observed.

A factor that makes immediate comparisons between our results and previously published studies difficult is the differences in the degree and duration of hypoglycemia. Besides, many of them have relied on fixation by immersion which inevitably produces artifacts. Only a few have applied comparable fixation by perfusion (Brierley et al. 1971; Myers and Kahn 1971). However, Brierley used FAM as a fixative which is inferior to GA for preservation of cellular details as is also formalin used by Myers and Kahn. Furthermore, the differences in embedding media influence the results and their interpretation as shown above. Brierley (1976) considered microvacuolation a characteristic nerve cell alteration also in hypoglycemia similarly as in anoxia and ischemia. However, microvacuolation is virtually absent in our GA-fixed material, a finding which was further ascertained in our electron microscopic study (Kalimo et al. 1980). Thus, both the biochemical and light microscopical results indicate a different pathogenesis of the nerve cell injury in hypoglycemia as opposed to anoxia-ischemia, despite the fact that these two conditions have the gross energy failure in common.

The two types of nerve cell injury do not seem to be different stages of one and the same reaction to the hypoglycemia. Thus, although we cannot exclude the possibility of a rapid transition between the two cell types the present results give no indication that such transitions occur. The type II neuronal injury is evidently less severe and most often reversible, since practically no such neurons were visible during the restitution. On the other hand, many type I injured neurons seem to be irreversibly damaged since such angulated condensed neurons were still present in all animals with restitution, even though their relative number did decrease during the restitution. This conclusion agrees well with our biochemical data. Thus, despite the remarkable recovery after 30 or 60 min

hypoglycemia there was a subnormal tissue ATP concentration and a reduced adenine nucleotide pool, less than complete restoration of the amino acid pool as well as moderate elevations of lactate concentration and lactate/pyruvate ratio. All of these changes are well comparable with an irreversible damage to some cells.

It is interesting that, following long periods of a severe hypoglycemia, many neurons displayed no or only minor morphological alterations, while nearby neurons showed extensive changes. Furthermore, there was certain predilection of the type of injury developing in different neurons. The small neurons, especially in layer 3, seemed to be more often affected by type I injury, whereas in the larger neurons, especially in layers 5 and 6, the type II injury was more common. There were also regional differences, e.g., neurons in the supply area of ACA were less readily affected. This indicates some kind of selective vulnerability, which may depend on the functional activity of the neurons, more active cells being exhausted earlier, or on the metabolic characteristics of the neuron, some having greater stores or slower consumption of substrates. Notably, neurons in the arterial border-zones were not selectively vulnerable, which is a further indication of the difference between the hypoglycemia and anoxic-ischemic nerve cell injury.

Acknowledgements. Supported by grants from the Swedish Medical Research Council (project Nos. B80-12X-03020-11C and 14X-263), from Turun Yliopistosäätiö (Turku, Finland), from USPHS (grant No. 5 R01 NS 07838) and from Anna Lisa and Sven-Erik Lundgren's Foundation for Medical Research. The skilled technical assistance from Mr. Ake Petterson, Mr. Christer Tengvar, Ms. Benita Andersson, Ms. Aira Mäkipää, and Ms. Kerstin Beirup is gratefully acknowledged. The photographic work was skilfully performed by Mr. Mauno Lehtimäki.

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Received December 7, 1979 / Accepted December 24, 1979

Hypoglycemic Brain Injury

II. Electron-microscopic Findings in Rat Cerebral Cortical Neurons During Profound Insulin-induced Hypoglycemia and in the Recovery Period Following Glucose Administration

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Summary. Severe hypoglycemia was induced in rats by insulin. The brain was fixed *in situ* by perfusion after the spontaneous EEG had disappeared for 30 or 60 min or after recovery had been induced for 30 or 180 min by glucose injection. Samples from the cerebral cortex from the area corresponding to the previous metabolic studies were processed for electron microscopy. The light-microscopic finding of two different types of nerve cell injury, reported in a preceding communication (Agardh et al. 1980), was also verified at the ultrastructural level. The type I injury was characterized by cellular shrinkage, condensation of the cell sap and nuclei, and perineuronal astrocytic swelling. No swelling of mitochondria occurred. The slightly swollen type II injured neurons showed contraction of mitochondria, disintegration of ribosomes, loss of RER, and appearance of membrane whorls, while their nuclear chromatin remained evenly distributed. No transition from one type to the other was observed. Neither type of nerve cell injury in hypoglycemia was like that commonly seen in anoxic-ischemic insults indicating a different pathogenesis in these states despite the common final pathway of energy failure. The loss of endoplasmic membranes and disintegration of ribosomes suggests that these structures might be sacrificed for energy production in the absence of normal substrates. During recovery, though, the number of type I injured neurons decreased while some of the remaining ones appeared even more severely affected, suggesting irreversible damage. Type II injured neurons were no longer seen indicating reversibility of these changes.

Key words: Hypoglycemia — Nerve cell injury — Electron microscopy — Rat cerebral cortex

cortex (Agardh et al. 1978), and structural alterations primarily in cortical neurons (Lawrence et al. 1942; Brierley et al. 1971; Myers and Kahn 1971). We have started a series of investigations on the morphological changes in rat brain caused by severe hypoglycemia of variable duration. Our aim is to correlate the altered structure with the biochemical and functional changes observed in the same model in order to reveal some of the pathogenetic mechanisms involved in hypoglycemic brain injury. In the preceding paper (Agardh et al. 1980) we described the light microscopic features of two different types of neuronal changes: Type I injury, which was considered irreversible in its severe form, was light microscopically characterized by angulated, darkly stained neurons with irregular outline resulting from condensation of cytoplasm and nuclei, and from perineuronal vacuolation. Type II injury, which seemed to be reversible, caused slight swelling of the neurons with subplasmalemmal, intracytoplasmic vacuolation or clearing (depending on the histotechnique used) but without nuclear alterations.

Our light microscopic findings and their interpretation (Agardh et al. 1980) differed to a significant degree from some previously published results (e.g., Brierley 1976). Among other things our morphological results, corroborated by biochemical data (Lewis et al. 1974; Norberg and Siesjö 1976; Agardh et al. 1978), suggested a different pathogenesis in hypoglycemic as opposed to anoxic-ischemic nerve cell injury despite the common final pathway in the depletion of energy-rich compounds. To further clarify the pathogenesis of the hypoglycemic brain injury we have also performed an electron microscopic investigation on the rat cerebral cortex during hypoglycemia and in the recovery period thereafter. This paper concerns the ultrastructural characteristics of changes in the perikarya of neurons afflicted by type I and type II hypoglycemic nerve cell injury. As far as we know ultrastructural reports on this important form of brain injury have not been presented earlier.

Hypoglycemia of sufficient severity causes major derangement in the cellular energy state of the cerebral

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Material and Methods

Hypoglycemia was induced in male Wistar rats (270–405 g) as described in the preceding metabolic and light-microscopic paper, and the experimental groups were the same as in its morphological part (Agardh et al. 1980). A summary of the experimental groups is given in Table 1.

At the termination of the experimental procedure a rapid thoracotomy was performed and the animals were fixed by intravascular perfusion with 3% glutaraldehyde in 0.1 mol · l⁻¹ phosphate buffer at pH 7.4, 37 °C and at a pressure of 135 mm Hg. After the completion of the perfusion the brain was removed and kept in the same fixative until further processed. Tissue pieces from the posterior frontal cortex over the convexity (Fig. 1) were sampled and

sectioned into 500 µm slices perpendicular to the surface with a tissue sectioner (The Michle Lab. Engl., Gomshall, Surrey, England). The slices were postfixed in 2% osmium tetroxide in cacodylate buffer, en bloc stained with 2% uranyl acetate in maleate buffer, dehydrated in a graded series of ethanol and embedded in epon. Semithin sections stained with toluidine blue were used for orientation and selected

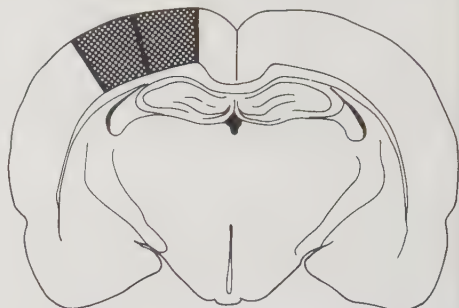


Fig. 1. The sampling of the cerebral cortex for electron microscopy. Pieces were taken from the area corresponding to that sampled for the biochemical analyses in the same model (Agardh et al. 1978, 1980)

Table 1. Summary of the experimental groups

Experimental group	Number of animals	Duration of isoelectric EEG	Duration of recovery
A (control)	5	—	—
B	5	30 min	—
C	3	60 min	—
D	5	30 min	30 min
E	3	60 min	180 min
F	3	60 min	60 min

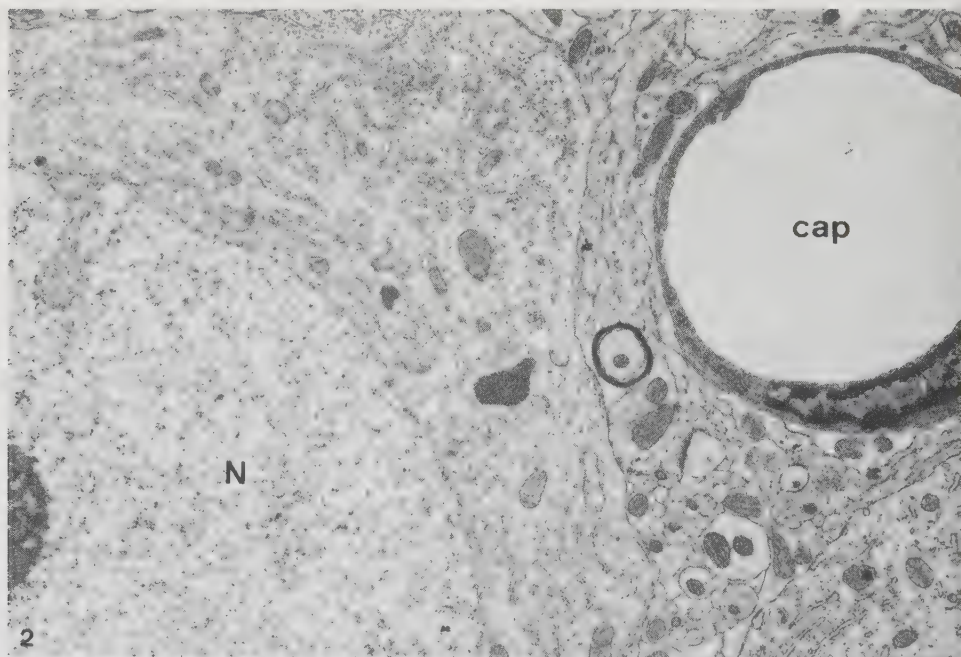


Fig. 2. Control animal. Two neurons and a capillary with the surrounding neuropil from the cerebral cortical layer 5 display the normal ultrastructure. Note the evenly distributed nuclear chromatin, numerous RER cisternae, ribosomal rosettes and moderate density of the mitochondria. *N* nucleus, *cap* capillary. ×10,000

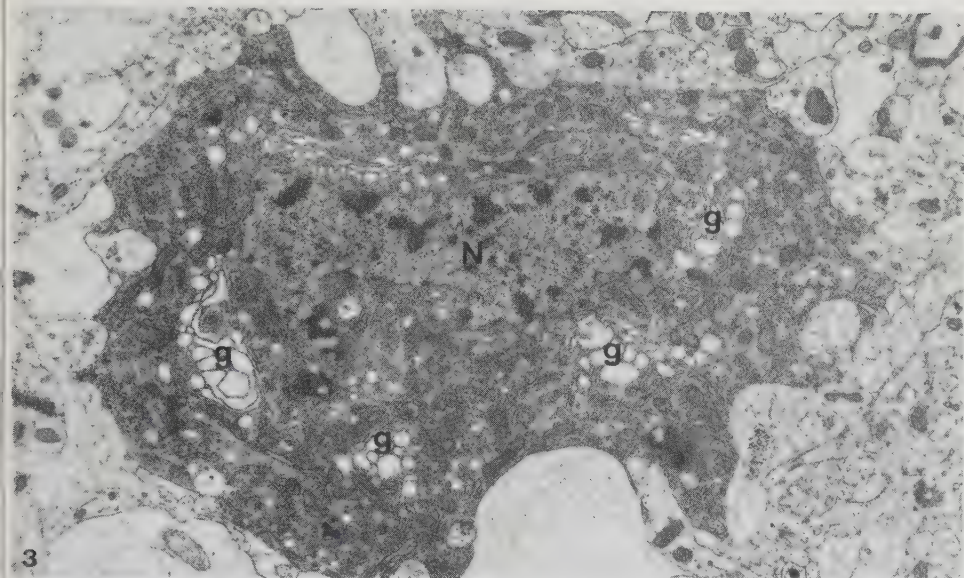


Fig. 3. A typical type I injured neuron in the cerebral cortical layer 3 from group B (30 min hypoglycemia). It is markedly condensed, darkly staining with dilated Golgi cisternae (g). Notably mitochondria are contracted. Perineuronal swollen processes bulge into the cytoplasm, which together with the dilated golgi cisternae might give these neurons microvacuolated outlook light microscopically. N nucleus. $\times 10,000$

areas were thinsectioned with a LKB I or a Porter-Blum MT-2 ultramicrotome, double stained with uranyl acetate and lead citrate and examined in a JEM 100C electron microscope.

Results

The fine structure of cerebral cortical neurons in our control animals corresponded to that generally considered normal and is illustrated in Fig. 2.

Alteration Occurring During Hypoglycemia

The light microscopic finding of two different types of injured neurons (Agardh et al. 1980) was verified at the ultrastructural level. In type I injury (Figs. 3 and 4) the neurons were condensed with somewhat irregular angulated form and corrugated plasma membrane. The cytoplasm was darkly stained with compact ribosomes tightly packed in clusters often around cisternae of rough-surfaced endoplasmic reticulum (RER). Mitochondria were most often elongated, condensed without an increase in the electron lucency of their inner matrix, and with no indications of extensive dilation as in certain type of anoxia-ischemia (Fig. 4). The Golgi cisternae were often dilated as were occasionally also RER cisternae (Fig. 3). The nuclei were also markedly

condensed and the nuclear membrane was irregularly corrugated (Fig. 3). Around those condensed neurons dilated watery cell processes nearly devoid of organelles were commonly seen. Most often they were interpreted as swollen perineuronal astrocytic processes. Sometimes they bulged into the shrunken cytoplasm so that in light microscopy an impression of intracytoplasmic vacuoles might well arise. As a change probably leading to type I injury some neurons showed increased stainability of the cyto- and karyoplasm without such pronounced shrinkage as in the full-blown type I injury (Fig. 5).

In type II injury (Figs. 6 and 7) the damaged neurons were more or less normal in shape or occasionally somewhat rounded. Characteristically, there was a decrease in the number of RER cisternae with concomitant disintegration of both RER-bound and free ribosomes. The ribosomes lost their orientation in rosettes as well as their compact structure with simultaneous decrease in size (Fig. 6–8). The remaining RER cisternae were commonly elongated, peripherally located. Most organelles gathered perinuclearly, thus resulting in clearing of the periphery, which light microscopically was seen already in the semithin epon-sections and which in JB-4 embedded sections gave the impression of subplasmalemmal vacuolization

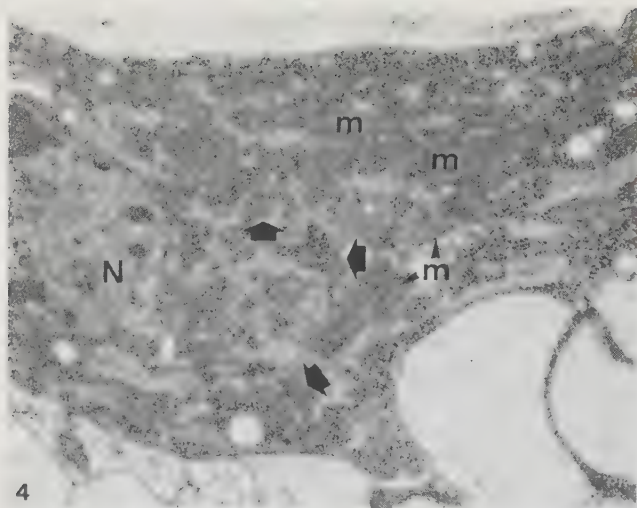


Fig. 4. A higher magnification of a type I injured neuron as above. The cyto- and karyoplasm are markedly condensed, compact ribosomes are in tightly packed clusters (*arrows*) between the contracted mitochondria (*m*) and RER cisternae. *N* nucleus. $\times 29,000$

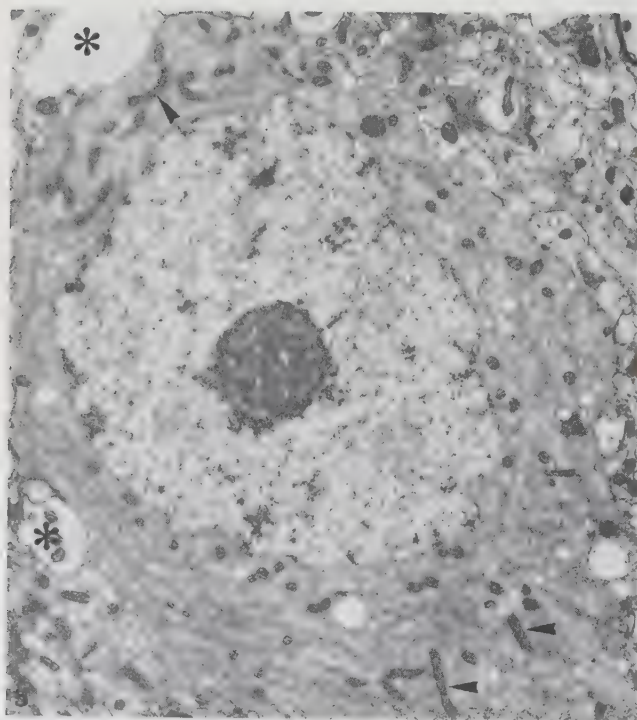


Fig. 5. Cerebral cortical layer 3-4 from group C (60 min). The neuron appears somewhat darker than normally evidently reflecting slight condensation. Ribosomes have lost their orientation in rosettes, mitochondria (*arrowheads*) appear contracted and there are a few dilated vacuoles (RER?). This most probably represents an early alteration leading to type I injury. Some perineuronal processes show incipient swelling (*asterisks*). $\times 9,200$

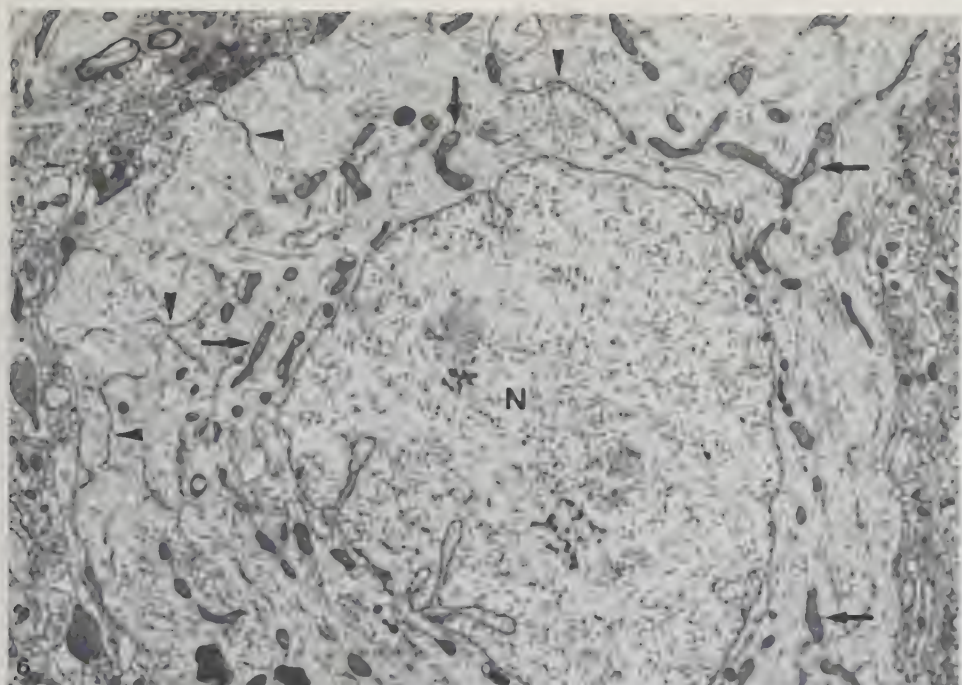


Fig. 6. A typical type II injured neuron in the cortical layer 5-6 from group C (60 min). The nuclear chromatin is evenly distributed. The cytoplasm stains light. RER cisternae (arrowheads) are long and narrow and their number appears decreased. Both free and membrane-bound ribosomes have lost their compact structure and ribosomal rosettes have disappeared: the cell sap appears thus finely granular. Mitochondria (arrows) are elongated and contracted. The periphery of the cytoplasm contains only few organelles resulting in the subplasmalemmal clearing/vacuolation seen in high microscopy. N nucleus. $\times 7,600$

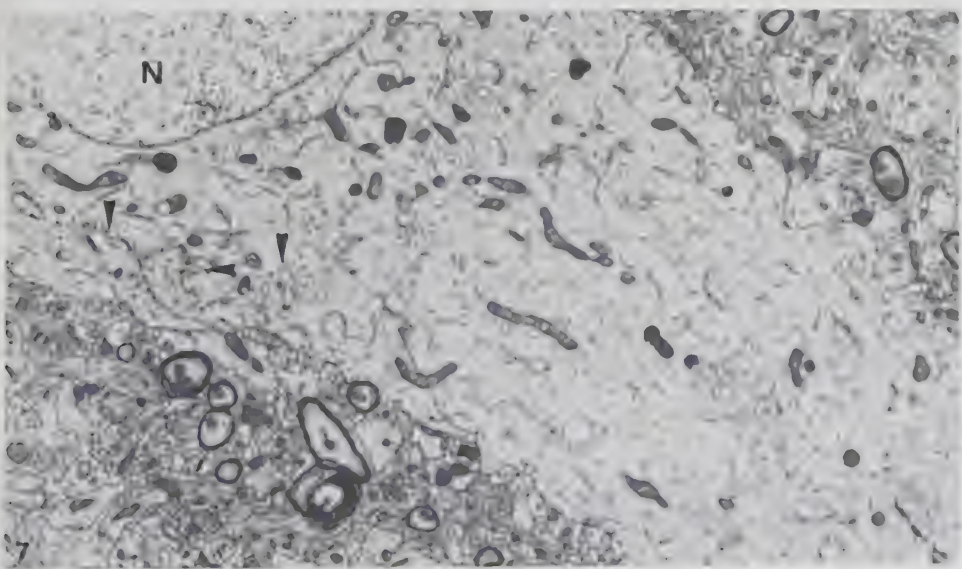


Fig. 7. Similar type II injured neuron as in Fig. 6. The structural alteration extends into the dendrite. Remarkably the surrounding neuropil looks quite normal. Membranous whorls (arrowheads) are seen in the peripheral cytoplasm. N nucleus. $\times 6,600$

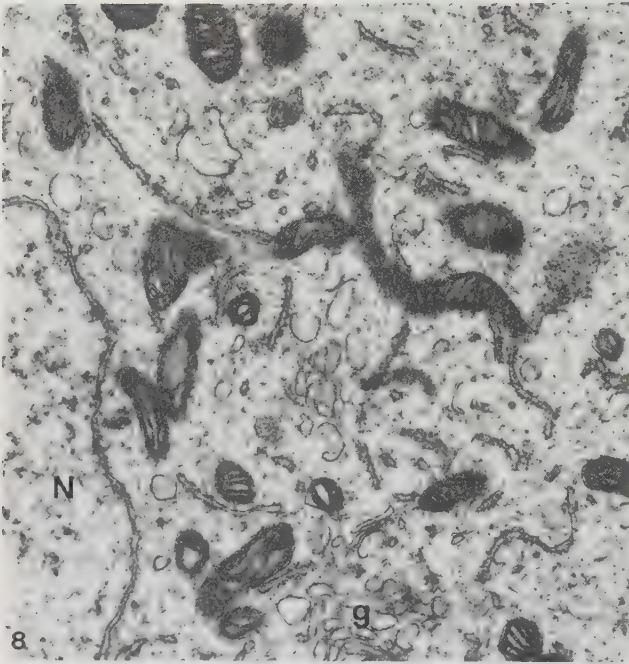


Fig. 8. A higher magnification of a similar type III injured neuron as in Figs. 6 and 7. The disintegration of the ribosomes and the contraction of the mitochondria become more apparent. $\times 24,000$



Fig. 9. Occasionally actual vacuoles (asterisk) are seen in the peripheral cleared cytoplasm of the type II injured neurons. Note the membranous whorls (arrowheads) and the typically contracted mitochondria. The neuron nearby appears virtually normal exemplifying the uneven distribution of the changes. Cortical layer 3 from group B (30 min). $\times 11,500$

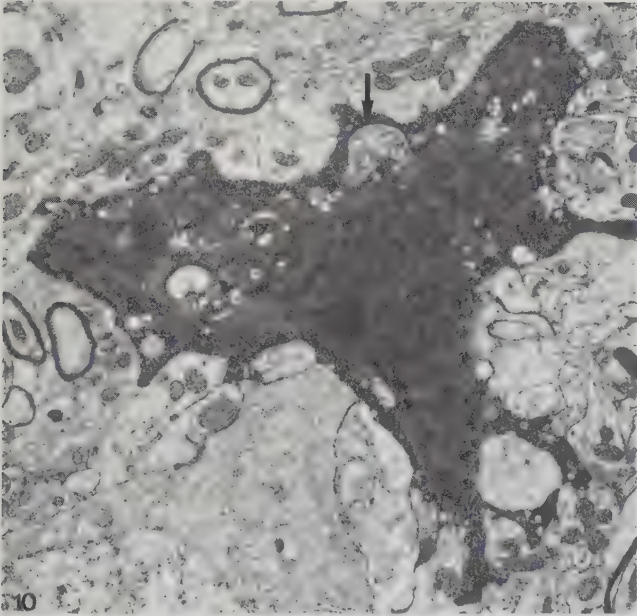


Fig. 10. Type I injured neuron from cortical layer 3 in group F (60 min hypoglycemia + 30 min restitution). The condensation is even more extensive than in Fig. 3. Furthermore, the inner matrix of mitochondria begins to show increased electron lucency (*arrow*). The perineuronal processes, though bulging into the neuronal cytoplasm, do not always appear swollen. This degree of injury may already be irreversible. $\times 8,200$

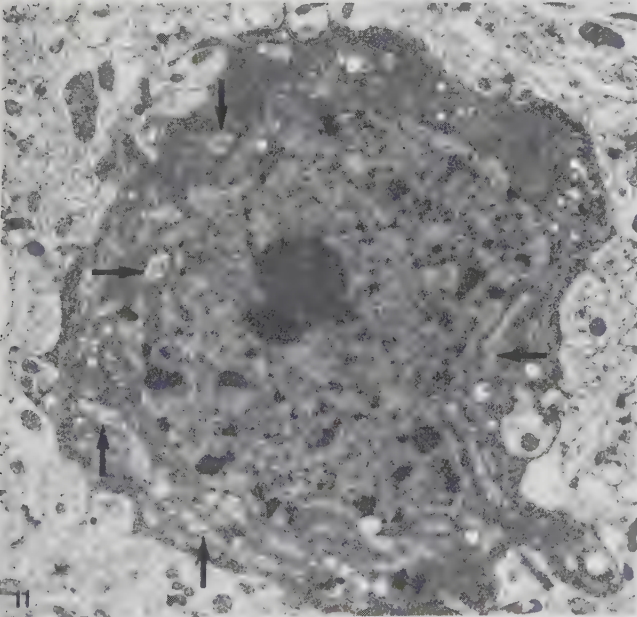


Fig. 11. This type I injured neuron from cortical layer 3 in group D (30 min + 30 min) appears quite similar as in animals without recovery. However, mitochondria (*arrows*) show increased electron lucency. Most perineuronal processes are not dilated. On the basis of the quantification on the light-microscopic specimens the injury of this severity may still be reversible. $\times 7,600$

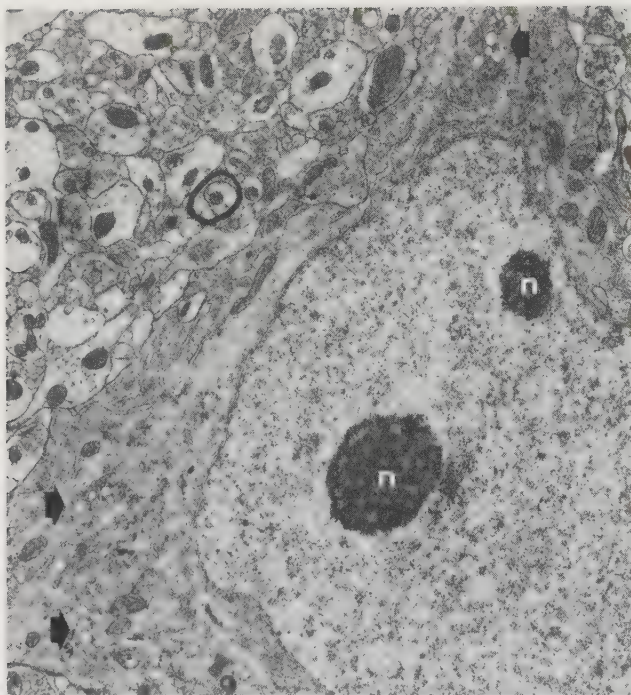


Fig. 12. Another neuron from group D (30+30 min) shows only slightly increased stainability with normal appearing mitochondria and prominent Golgi complexes (arrows). Two nucleoli (n) and more compact appearing ribosomes evidently indicate recovery from type I injury. $\times 8,300$

(Agardh et al. 1980). This change extended also into the neuronal dendrites (Fig. 7). Occasionally, actual vacuoles were seen in the peripheral cytoplasm (Fig. 9), where, in addition, membranous whorls became common with longer duration of the hypoglycemia (Figs. 7 and 9). The mitochondria were markedly contracted with elongated shape and darkly stained inner matrix (Figs. 6–8). The cisternae of the Golgi complexes were short and rounded in shape. Remarkably, the nuclei retained their normal shape and the chromatin remained evenly dispersed. Neither did the nucleoli show any striking changes.

Qualitatively, the changes in the neurons were similar after both 30 and 60 min hypoglycemia; they only became somewhat more accentuated with longer duration of the insult. The alterations were, however, very uneven in distribution and neurons with essentially normal appearance could be seen next to injured ones.

Alterations Occurring During Recovery

Neurons with type I injury were present in all animals with recovery, even though their number decreased as

presented in the preceding light microscopic paper (Agardh et al. 1980). They appeared relatively similar to those seen in animals without recovery, yet some neurons in group F (60+30 min) appeared even more severely injured indicating a continuing degenerative process (Fig. 10). Furthermore, the mitochondria in the remaining type I injured neurons usually showed increased electron lucency of their inner matrix (Figs. 10 and 11), which was not seen after hypoglycemia without recovery (Figs. 3 and 4). However, extreme mitochondrial ballooning with ruptured cristae was not seen during the recovery either. Notably, the swelling of the perineuronal processes appeared less prominent. Some cells during the recovery (Fig. 12) appeared fairly similar to those interpreted as an incipient type I injury (Fig. 5), with the exception of having less contracted mitochondria and more prominent Golgi complexes. These cells could well be recovering from the still reversible type I injury.

During recovery neurons with definite type II injury were no more seen. There were some neurons in which the RER was rather sparse and the ribosomes appeared

less compact than normally. These could be neurons recovering from the type II injury. In some of these neurons the Golgi complex appeared enlarged being composed of numerous small vesicles, and multivesicular bodies were fairly common. Notably contracted mitochondria, so characteristic of type II injury were not seen any more.

Discussion

The light microscopical finding of two different types of hypoglycemic nerve cell injury (Agardh et al. 1980) was also verified in the electron microscopic investigation. The type I injured neurons displayed generalized condensation of the cytoplasm and nuclei resulting in crowding of the organelles. In contrast to the early, extensive ballooning of neuronal mitochondria with ruptured cristae seen in anoxia-ischemia of the incomplete and/or transient type (Brown and Brierley 1973; Arsenio-Nunes et al. 1973; Garcia et al. 1977) the mitochondria in type I hypoglycemic injury rather appeared slightly contracted and only after recovery they showed moderately increased electron lucency of the inner matrix. Otherwise, the type I injured cells closely resemble the ischemically injured neurons as well as "dark neurons" seen in some other types of nerve cell injuries (Noack et al. 1971). Since this characteristic cell change occurs in different conditions it is evidently not specific for hypoglycemia. It should be emphasized that "dark neurons" may also occur as a fixation artefact. However, as discussed in the light-microscopic part of this series (Agardh et al. 1980), such cells are not seen in controls fixed in the same prompt and efficient way of intravascular perfusion. We therefore conclude that they represent a change present already at the terminating moment of the experiment.

The reduction in cell volume ('condensation') in type I injury remains unexplained. It has been suggested that volumetric changes in acute, lethal cell injury are related to an altered permeability of the plasma membrane (Trump and Arstila 1975). In most cells the depletion of cellular energy stores, and the ensuing decrease in the activity of the membrane-bound Na^+ , K^+ -ATPase, would result in influx of osmotic equivalents and water, which is seen as swelling of the cell and its organelles (Jennings et al. 1975; Trump and Arstila 1975). Although it has been suggested that the exceptional condensation of injured neurons in transient and/or incomplete cerebral anoxia-ischemia is due to the unique nature of the neuronal plasma membrane with its specific ion transport systems (Garcia et al. 1977) the mechanisms remain elusive. Obviously, although the special metabolic features of hypoglycemia (e.g. lack of cellular acidosis) may prevent distension

and rupture of mitochondria they do not prevent condensation of injured neurons.

The reorganization and reduction of RER cisternae and the disintegration of ribosomes in type II nerve cell injury resulted in cleared peripheral cytoplasm devoid of most organelles. Actual vacuolation was, however, not very common. Thus, the falsity of the subplasmalemmal vacuoles seen in paraffin and JB-4 embedded material (Agardh et al. 1980) was verified also at the ultrastructural level. The type II injury may represent a characteristic hypoglycemic nerve cell damage, since we are not aware of exactly the same ultrastructural changes in other cell injuries. The absence of oxygen also results in morphological disintegration of ribosomes (Kalimo et al. 1977) and inhibition of polysomal protein synthesis (Yanagihara 1975), but the mitochondrial and nuclear changes are different. Fragmentation and dispersion of RER and its ribosomes occurs in the retrograde axon reaction, but the ribosomes do not disintegrate in the same way and the RER cisternae in hypoglycemia were rather long tubules than small vesicles as in the chromatolysis of the axon reaction (Price and Porter 1972).

Interestingly, somewhat similar RER and ribosomal alterations were recently reported in brindled mouse (Yajima and Suzuki 1979). This mouse is a mutant with clinical and biochemical features closely similar to human kinky hair syndrome, in which defective copper absorption possibly results in the deficient activity of mitochondrial copper containing enzymes (cytochrome oxidase, etc.). In brain this causes degeneration of neurons which have, besides the comparable RER and ribosome alteration, large abnormal mitochondria.

It is surprising that ribosome disintegration occurred so early in the hypoglycemic period. One reason could be the fact that the ribosomeoities are used for energy production. The morphological disintegration was also biochemically substantiated as marked reduction of RNA was measured in cerebral cortex after 30–60 min of hypoglycemia (Agardh et al., in prep.). The reduction of RER and accumulation of membrane whorls, characteristic of the type II injury, are probably due to degradation of membrane lipids. Possibly RER constitutes sacrificable membranes which could be utilized for energy production. Measurements of cerebral metabolic rates in severe hypoglycemia clearly show that oxygen consumption may grossly exceed glucose consumption over long periods (Pappenheimer and Setchell 1973; Norberg and Siesjö 1976). During short intervals, the missing (endogenous) substrates may be provided by existing stores of carbohydrates and amino acids (Norberg and Siesjö 1976). However, there is evidence that protracted hypoglycemia causes mobilization of structure-bound substrates, such as

phospholipids (Hinzen et al. 1970). It is conceivable that when this occurs, irreversible structural damage is imminent.

Condensation of mitochondria, even in advanced type II injury, was another characteristic finding. This feature also contrasts with the anoxic-ischemic cell injury in which moderate to excessive high amplitude swelling occurs following the early transient condensation (Trump and Arstila 1975; Garcia et al. 1977; Kalimo et al. 1977). Conformational changes are seen in mitochondria depending on their metabolic state. It is of interest that marked condensation of isolated mitochondria was described by Hackenbrock (1966) and Hackenbrock et al. (1971) in state II, i.e., in mitochondria incubated in the absence of substrate with oxygen available. It remains to be shown whether the absence of marked swelling of mitochondria is related to the substrate deficiency or if it reflects the fact that energy is continuously produced, albeit at insufficient amounts to maintain cellular function (and energy state).

In conclusion, the present electronmicroscopic study verified the presence of two types of hypoglycemic nerve cell injury and further characterized their structural features. In many respects, the cell changes observed differ from anoxic-ischemic nerve cell injury. That, in conjunction with the biochemical data, implies that the pathogenesis of nerve cell injury in hypoglycemia is different from that in anoxia-ischemia, with which it has previously been equated.

Acknowledgements. Supported by grants from the Swedish Medical Research Council (project Nos. B80-12X-03020-11C and 14X-263), from Turun Yliopistosäätiö (Turku, Finland), from USPHS (grant No. 5 RO1 NS 07838) and from Anna Lisa and Sven-Erik Lundgren's Foundation for Medical Research. We are grateful for the skilled technical assistance of Ms. Kerstin Beirup, Ms. Elisa Saarinen, Ms. Paula Merilähti, and Ms. Madeleine Jarild; the photographic work of Mr. Mauno Lehtimäki; and for the secretarial help of Ms. Kaarina Jokinen.

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Received December 7, 1979/Accepted January 15, 1980

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INFLUENCE OF SEVERE HYPOGLYCEMIA ON MITOCHONDRIAL AND PLASMA
MEMBRANE FUNCTION IN RAT BRAIN

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ABSTRACT

Previous experiments have shown that severe hypoglycemia disrupts cerebral energy state in spite of an upheld cerebral oxygen consumption, suggesting uncoupling of oxidative phosphorylation. Other studies have demonstrated that hypoglycemia leads to loss of cerebral cortical phospholipids and phospholipid-bound fatty acids. The objective of the present study was, therefore, to study respiratory characteristics of brain mitochondria and to correlate respiratory activity to mitochondrial phospholipid composition. To that end, mitochondria were isolated after 30 or 60 min of hypoglycemia with ceased EEG activity, as well as after a 90 min recovery period, and their resting (state 4) and ADP-stimulated (state 3) oxygen consumption rates were measured. In separate experiments, mitochondria were isolated for analyses of phospholipids and phospholipid-bound fatty acids.

After 30 min of hypoglycemia state 3 respiration decreased without any increase in state 4 respiration or change in ADP/O ratio. This decrease, which occurred with glutamate plus malate, but not with succinate, as substrates, was partly reversed by addition of bovine serum albumin and KCl. Chemical analyses of isolated mitochondria did not reveal changes in their phospholipid or fatty acid content. The results thus failed to account for the dissociation of cerebral energy state and oxygen consumption. However, it is tentatively concluded that uncoupling may occur in vivo due to accumulation of free fatty acids and "futile cycling" of K^+ and Ca^{2+} .

After 60 min of hypoglycemia, a moderate decrease in state 3 respiration was observed also with succinate as substrate, and there was some decrease in ADP/O ratios in KCl-containing media. However, the changes in ADP/O ratios were more conspicuous during recovery; in addition, state 4 respiration increased significantly. It is concluded that changes in mitochondrial function after 30 min of hypoglycemia are potentially reversible but that true mitochondrial failure develops in the recovery period following 60 min of hypoglycemia. This conclusion was corroborated by results demon-

strating incomplete recovery of cerebral energy state.

Since it seemed difficult to explain failure of return of electrophysiological function after 60 min of hypoglycemia solely by mitochondrial dysfunction, plasma membrane function was assessed by measurements of extracellular potassium activity ($[K^+]_e$). The results showed that whereas $[K^+]_e$ remained close to control in the recovery period following 30 min of hypoglycemia it rose progressively during recovery following 60 min of hypoglycemia. It is concluded that increased ion conductance and/or inhibition of Na^+-K^+ activated ATPase could contribute to the permanent loss of spontaneous or evoked electrical activity.

INTRODUCTION

Severe hypoglycemia is associated with clinical coma and with loss of spontaneous electroencephalographic (EEG) activity (Samson et al. 1959, Creutzfeldt and Meisch 1963, Mergenhagen et al. 1968, Lewis et al. 1974b, Feise et al. 1976). At these degrees of hypoglycemia several groups have demonstrated gross derangement of cerebral energy state with low concentrations of PCr and ATP, and increased concentrations of ADP and AMP (Tews et al. 1965, Hinzen and Müller 1971, Lewis et al. 1974a and b, Feise et al. 1976, Norberg and Siesjö 1976, Agardh et al. 1978) with an associated release of potassium from cells (Astrup and Norberg 1976).

During hypoglycemia, the cerebral oxygen consumption (CMR_{O_2}) is not reduced to the same extent as the cerebral metabolic rate for glucose (CMR_{gl}) (Kety et al. 1948, Eisenberg and Seltzer 1962, Della Porta et al. 1964, Gottstein and Held 1967, Pappenheimer and Setchell 1973, Norberg and Siesjö 1976, Agardh et al. 1981), indicating that endogenous substrates are being consumed. Such substrates include glycolytic metabolites as well as citric acid cycle intermediates and associated amino acids (Goldberg et al. 1966, Lewis et al. 1974a, Norberg and Siesjö 1976, Agardh et al. 1978). However, several studies demonstrate that hypoglycemia reduces tissue concentrations of structural components as well, notably phospholipids and RNA (Randall 1940, McGhee et al. 1951, Knauff et al. 1961, Hinzen et al. 1970, Agardh et al. 1981).

If the hypoglycemic state is sufficiently prolonged, irreversible neuronal damage develops (Brierley et al. 1971, Myers and Kahn 1971, Agardh et al. 1980, Kalimo et al. 1980). Although the mechanisms involved are not known it is tempting to conclude that the derangement of cerebral energy state and/or oxidative breakdown of cellular membrane structures are responsible. Two findings provided the immediate background to the present study. First, CMR_{O_2} is upheld at a time when cerebral energy state is grossly deranged, indicating increased energy demands or uncoupling of oxygen consumption and mitochondrial ATP production (Norberg and Siesjö 1979, see al-

so Nilsson et al. 1981). Second, in the same model hypoglycemia reduced cerebral cortical concentrations of phospholipid-bound fatty acids by about 10% (Agardh et al. 1981). Accordingly, we decided to study respiratory functions of isolated mitochondria and to evaluate changes in mitochondrial phospholipids and phospholipid-bound fatty acids.

As the results will show, 30 min of severe hypoglycemia only reduced state 3 (ADP-stimulated) respiration of mitochondria with glutamate plus malate as substrates, and no evidence was obtained of loss of coupling. Furthermore, the results failed to reveal significant breakdown of mitochondrial phospholipids. Our continued work was, therefore based on two additional findings: when hypoglycemia is prolonged from 30 to 60 min, the number of irreversibly damaged cells seems to increase substantially (Agardh et al. 1980, Kalimo et al. 1980) and recovery of neurophysiological functions is markedly depressed (Agardh and Rosén in preparation). Prolongation of hypoglycemia to 60 min revealed that isolated mitochondria were now loosely coupled, and measurements of labile tissue metabolites gave evidence of a permanently deranged cerebral energy state. However, since it could not be concluded that failure of energy production alone was the cause of the lack of return of electrophysiological functions we measured extracellular potassium concentration in the recovery period, following 30 and 60 min of severe hypoglycemia.

METHODS

Animals, operative and sampling techniques

Male Wistar rats (305-440 g) of a SPF Wistar strain (Møllegaard Avlslaboratorium, Copenhagen) were fasted overnight before the experiments but had free access to tap water. One hour before operation they were given an intraperitoneal injection of insulin (Insulin Novo Actrapid^(R), Novo Industri AB) in a dose of 40 IU·kg⁻¹. The insulin was dissolved in 0.75 ml of Krebs-Hensleit solution before injection. Control animals were given 0.75 ml of this solution. Analgesia was induced with 80% N₂O and 20% O₂ and incision sites were in-

filtrated with lidocaine ($10 \text{ mg}\cdot\text{ml}^{-1}$), $0.4\text{--}0.5 \text{ ml}$ per animal. The animals were then immobilized with an i.p. injection of 1.5 mg of tubocurarine chloride, tracheotomized and ventilated with a Starling type respirator, which was adjusted to give an arterial PCO_2 of $30\text{--}40 \text{ mm Hg}$. The body temperature was maintained at around 37°C . One femoral artery was cannulated for injections. The electrocorticogram (EEG) was continuously recorded in all animals from gold-plated copper bolts inserted into the skull bone in the fronto-parietal region (bipolar leads). After the operative procedures had been completed, the animals were heparinized (100 IU i.v.). Repeated arterial samples were anaerobically taken from the arterial catheter into glass capillaries for determination of pH, PCO_2 , and PO_2 , or collected directly in liquid nitrogen for later analyses of glucose.

Experimental groups

There were four series of animals, each with its own control group. In series A, the respiratory function of isolated mitochondria was evaluated either after 30 ($n = 4$) or after 60 ($n = 4$) min of severe hypoglycemia (with ceased EEG activity) or after a 90 min recovery period, following either 30 ($n = 6$) or 60 ($n = 6$) min of hypoglycemia. In this series, and the following one (series B) the experiment was terminated by decapitation of the animals, and isolation of mitochondria (see below). In the recovery groups, 0.5 ml of a 50% (w/v) glucose solution (in saline) was injected i.v. following the end of the hypoglycemic periods. This was followed by a slow i.v. infusion ($1 \text{ ml}\cdot\text{h}^{-1}$) of the glucose solution. Series B was similar but instead of studying the respiratory function of the isolated mitochondrial fraction we analysed phospholipids and phospholipid-bound fatty acids after 30 min of hypoglycemia ($n=6$). Series C. Since results on mitochondrial function in the recovery period after 60 min of hypoglycemia gave evidence of loose coupling, cerebral cortical tissue was frozen in situ for assessment of tissue energy state after 180 min of recovery following hypoglycemia with an isoelectric EEG for 60 min ($n=6$). In series D, finally, recovery was induced following a 30 ($n=4$) or 60 ($n=6$) min period of hypoglycemia and extracellular $[\text{K}^+]_e$ was recorded. In the 60 min group, the brain tissue of two animals was frozen in situ

at the end of the experiments and cortical tissue around the electrode tip, and a corresponding area from the opposite hemisphere, were later analysed for labile organic phosphates (see Results and Discussion).

In the hypoglycemic groups, the periods of anaesthesia were around 3 hrs and, in the recovery periods, around 4 hrs. Accordingly, control groups were maintained under anaesthesia for 3-4 hrs.

Isolation of mitochondria

At the ends of the experimental periods the rats were decapitated and the brains quickly removed from the skull. Brain mitochondria were then isolated by the method of Clark and Nicklas (1970) with the modifications described by Mela and associates (see Ginsberg *et al.* 1977, Rehncrona *et al.* 1979).

Measurements of mitochondrial respiratory activity

Oxygen uptake was measured polarographically in a closed and magnetically stirred chamber (volume 0.63 ml) with an oxygen electrode operating at 24°C. The electrode was calibrated with gas mixtures of known composition. The incubation medium contained 0.225 M mannitol, 0.075 M sucrose, 1 mM EGTA, 0.01 M H_3PO_4 and 0.01 M HCl and was adjusted to pH 7.4 with Tris (Trizma^(R) Base, Sigma). Henceforth, this basic solution will be denoted as MSTP_i. In some experiments, 100 mM KCl, 2.5 % bovine serum albumin (BSA), or 100 mM KCl plus 2.5% BSA, were added to the incubation medium. A 10 μl aliquot of the mitochondrial suspension (containing 250-300 μg of protein) was used for each determination. Twenty μl of 0.5 M malate plus glutamate and 10 μl of 1 M succinate, were used as substrates, respectively. When succinate was used, the electron transfer from NAD to cytochrome *b* was blocked by adding 3 μl of 1 M Rotenone prior to succinate administration. State 3 respiratory activity was initiated by adding 3 μl of 85 mM ADP. All measurements were performed in duplicate. Since the ratio of cytochrome *a-a*₃ activity to protein concentration remained unchanged in all groups, respiratory activities were related to mitochondrial protein content.

Analytical techniques

The pH, PCO_2 , and PO_2 in arterial blood were measured immediately after sampling with microelectrodes operated at 37°C (Radiometer, Copenhagen and Eschweiler & Co, Kiel) with appropriate temperature corrections.

For analysis of mitochondrial phosphorus content, individual phospholipid classes, and total fatty acid content 200 μl of the mitochondrial suspension were mixed with 3.0 ml of chloroform-methanol (C:M 1:1 v/v), with 50 μl BHT (2,6 di-tert-butyl-p-cresol) added as antioxidant, and kept at -80°C until extraction. The mixture was left in room temperature for 60 min, then centrifuged at $2.500 \times g$ for 10 min. The tissue residue was then extracted once more with 4.0 ml C:M (3:1 v/v) and 0.1 ml H_2O and was left in room temperature for 30 min and then filtered through glass filter retaining particles bigger than 90 μm . The combined extracts were shaken with 2.0 ml of 1% NaCl in 0.01 M HCl and centrifuged at $2.500 \times g$ for 10 min. The upper layer was discarded, the lower layer taken to dryness in a stream of N_2 . The lipids were then dissolved in 3.0 ml C:M (2:1, v/v). Ten μl aliquots (in triplicate) and 0.3 ml were taken for determination of total phosphorus and total fatty acids (FA), respectively. Two ml were taken for separation of individual phospholipids by thin-layer chromatography and subsequent measurement of lipid phosphorus. The FA were transesterified and the methyl-esters obtained were separated with gas-liquid chromatography. For further details, see Agardh et al. (1981). Mitochondrial protein concentration was measured according to Lowry et al. (1951). Cytochrome (a + a_3) was determined with a dual wavelength spectrophotometer (Aminco D-W 2) at 445-460 nm as the difference in optical density between the fully oxidized and reduced states. For measurements of labile metabolites the tissue was frozen in situ (Pontén et al. 1973) and a 100-200 mg cortical sample was dissected and extracted with HCl-methanol at -20°C . The further processing of the samples and the measurements of metabolites with enzymatic, fluorometric techniques, have been described in previous communications (see Agardh et al. 1978).

Measurement of extracellular $[K^+]$

After about 60-90 min of recovery following 30 or 60 min respectively, of hypoglycemia with an isoelectric EEG, a craniotomy was performed. The head was then immobilized and a double-barrelled K^+ microelectrode (tip diameter 1-2 μm) was stereotactically inserted into the cerebral cortex through a small slit in the dura. Thereafter the K^+ potential was continuously recorded. The construction and use of the K^+ electrode has been previously described (Astrup and Norberg, 1976).

Calculations

The concentration of cytochrome (a + a₃) was calculated as the difference in optical density at an extinction coefficient of 160 mM⁻¹.cm⁻¹. The oxygen consumption rate was calculated as nmol O₂ consumed per mg of mitochondrial protein per min. Respiratory control ratio (RCR) was calculated as state 3 divided by state 4 respiratory activities.

Statistical differences were evaluated with Mann-Whitney U-test (two-tailed), and Student's t-test.

RESULTS

1. Arterial blood glucose concentrations and physiological parameters.

Profound hypoglycemia was defined as the period with ceased EEG activity ("isoelectric EEG"). Previous work has shown that EEG becomes isoelectric when the arterial blood glucose decreases to or below 1 $\mu mol \cdot g^{-1}$. In the present study, the arterial blood glucose concentration of control animals varied between 5.6 and 9.4 $\mu mol \cdot g^{-1}$ (7.2 ± 1.2 ; mean $\pm$ S.E.M.), and between 1.2 and 0.2 (0.74 ± 0.03) $\mu mol \cdot g^{-1}$ during hypoglycemia with an abolished EEG activity. During the recovery period, blood glucose was normalized and not significantly different from control ($p > 0.05$). In all experiments hypotension was excluded by continuous blood pressure recording. The arterial blood pressure was usually well over 100 mm Hg. The arterial P_{O_2} was kept at around 100 mmHg and P_{CO_2} between 30 and 40 mmHg.

2. Mitochondrial function and phospholipid composition.

Since the discrepancy between CMR_{O_2} and cerebral energy state is largest during the first 15 min of hypoglycemic coma, (Norberg and Siesjö 1976, Agardh et al. 1981) we initially concentrated our efforts on mitochondrial function and phospholipid composition after 30 min. Following a brief characterization of the mitochondrial fraction we will, therefore, describe the 30 min groups before considering the 60 min series.

a. Mitochondrial protein yield and cytochrome a-a₃ content. Since hypoglycemia is a pathological condition which might well influence the sedimentation properties of the mitochondria we estimated the total protein yield in the mitochondrial fraction by multiplying the total amount of isolated suspended mitochondria (μ l) with the protein content ($g \cdot l^{-1}$). As Table 1 shows, hypoglycemia of 30 and 60 min duration neither influenced the protein nor the cytochrome a-a₃ content.

Table 1. Total protein yield and cytochrome (a + a₃) content in rat cerebral mitochondria from controls, during hypoglycemia and in the recovery period following glucose administration.

	Control	Hypoglycemia with ISO-EEG		Recovery 90 min following ISO-EEG	
		30 min	60 min	30 min	60 min
Protein total (mg)	13.3 $\pm$ 0.6	11.6 $\pm$ 0.6	13.5 $\pm$ 0.3	12.2 $\pm$ 0.3	14.5 $\pm$ 0.6
Cytochrome (a+a ₃) content (nmol)	2.7 $\pm$ 0.2	2.4 $\pm$ 0.2	2.7 $\pm$ 0.1	2.5 $\pm$ 0.1	3.1 $\pm$ 0.1
Cytochrome (a+a ₃) (nmol/mg prot.) ³	0.197 $\pm$ 0.004	0.207 $\pm$ 0.008	0.200 $\pm$ 0.009	0.200 $\pm$ 0.006	0.214 $\pm$ 0.008

The values are means $\pm$ S.E.M. No statistical differences between the experimental groups and control. (Mann-Whitney U-test)

Accordingly, the cytochrome a-a₂ activity per unit weight of protein remained constant. This pattern also prevailed in the recovery groups.

Table 2. State 4 and state 3 respiratory activities ($\text{nmol} \cdot \text{O}_2 \cdot \text{mg mitochondrial protein}^{-1} \cdot \text{min}^{-1}$), respiratory coefficient rates (RCR) and ADP/O ratios in rat cerebral mitochondria in controls (C), during hypoglycemia with an isoelectric EEG for 30 min (H) and after 90 min of recovery following hypoglycemia with an isoelectric EEG for 30 min(R).

Added substrate	State 4			State 3			RCR			ADP/O		
	C	H	R	C	H	R	C	H	R	C	H	R
<u>Malate+glutamate</u>												
+ MSTPi	14.5 +1.1	11.7 +0.9	13.8 +1.9	54.0 +2.9	30.9*** +3.3	60.5 +8.3	3.9 +0.2	2.6** +0.2	4.5 +0.3	2.8 +0.1	3.1 +0.3	2.6 +0.1
+ MSTPi+2.5% BSA	13.0 +1.4	74.0 +2.6		74.0 +2.6			5.9 +0.7			2.4 +0.1		
+ MSTPi+100mM KCl	14.9 +1.4	62.3 +3.3		62.3 +3.3			4.7 +0.1			2.5 +0.1		
+ MSTPi+2.5% BSA +100 mM KCl	10.4 +0.6	10.9 +0.3	11.9 +0.9	69.0 +3.5	52.9* +2.1	83.8 +7.7	6.8 +0.2	4.9*** +0.2	7.2 +0.4	2.6 +0.1	2.5 +0.1	2.4 +0.1
<u>Succinate</u>												
+ MSTPi	16.6 +0.7	15.0 +0.9	16.2 +1.2	57.0 +1.6	55.5 +0.9	62.1 +3.9	3.5 +0.2	3.8 +0.2	4.0 +0.3	1.8 +0.1	1.7 +0.0	1.7 +0.1
+ MSTPi+2.5% BSA	15.9 +1.0			67.1 +5.9			4.3 +0.5			1.6 +0.1		
+ MSTPi+100mM KCl	15.1 +0.5			58.4 +1.8			3.9 +0.2			1.7 +0.0		
+ MSTPi+2.5% BSA + 100mM KCl	13.9 +2.0	12.8 +0.9	13.1 +0.8	73.7 +2.2	68.6 +4.6	75.9 +2.7	5.3 +0.3	5.4 +0.4	5.8 +0.2	1.9 +0.1	1.8 +0.0	1.8 +0.1

The values are means + S.E.M. * $p < 0.05$; ** $p < 0.02$; *** $p < 0.002$ (Mann-Whitney U-test)

b. Hypoglycemia of 30 min duration. Table 2 gives the state 4 and state 3 respiratory rates, the RCR values, and the ADP/O ratios at the end of the hypoglycemic period, as well as after 90 min of recovery. In the control group, addition of BSA and/or KCl somewhat reduced state 4 and increased state 3 respiration. As a result, RCR increased significantly. The results indicate that the effect was primarily due to BSA. However, the combination of BSA and KCl failed to alter the ADP/O ratio.

Hypoglycemia had no influence on state 4 respiration but significantly reduced state 3 respiration when glutamate plus malate served as substrates. Addition of BSA + KCl considerably ameliorated this reduction but could not prevent it altogether. As a result, RCR values were significantly reduced. However, ADP/O ratios remained unchanged. Somewhat unexpectedly, hypoglycemia failed to influence state 3 respiration or RCR values when succinate was used as substrate.

During the recovery period, the inhibition of state 3 respiration was reversed and, since state 4 respiration remained unchanged, the RCR values were normalized as well.

In view of the moderate changes in respiratory functions induced by hypoglycemia the question arose whether the hypoglycemia affected mitochondrial phospholipid content and composition. As previously described from this laboratory, hypoglycemic coma of this duration leads to a decrease in the cerebral cortical phospholipid concentrations by 8 per cent and a decrease in total fatty acid concentration by 10 per cent (Agardh et al. 1981). Results on mitochondria are shown in Table 3. In accordance with previous reports (see Abood 1969) we found that the mitochondria contained a higher proportion of unsaturated than saturated fatty acid (60 versus 40 per cent, as compared with 53 versus 47 per cent in cortex, see Agardh et al. 1981). Hypoglycemia did not alter the mitochondrial contents of total fatty acids, major fatty acid classes, phospholipid phosphorus, or individual phospholipid classes. It should be noted that the concentrations of polyenoic fatty acids (20:4 and 22:6) remained constant during hypoglycemia. (The values were 14.7 ± 0.3 and 18.1 ± 0.2 per cent, respect-

ively). The results thus indicate that the phospholipids and fatty acids lost from the cerebral cortex are not of mitochondrial origin (see Discussion).

Table 3. Major fatty acids and phospholipid phosphorus concentrations in rat cerebral mitochondria in controls and during hypoglycemia with an isoelectric EEG for 30 min.

	Control	Hypoglycemia
Fatty acids total ($\mu\text{mol/g}$ mitoch.protein)	1245 $\pm$ 72	1193 $\pm$ 70
Per cent distribution:		
Saturated ^{a)}	43.6	44.0
Monounsaturated ^{b)}	21.4	21.5
Polyunsaturated ^{c)}	34.9	34.5
Phospholipids total ($\mu\text{mol/g}$ mitoch.protein)	594 $\pm$ 11	595 $\pm$ 8
Per cent distribution:		
SM	3.0	2.8
CPG	38.5	38.4
IPG + SPG	12.6	11.6
EPG	45.6	46.7

The values represent means + S.E.M.

a) Including 16:0, 18:0 and 20:0. b) Including 16:1, 18:1 and 20:1.

c) Including 18:2, 20:3, 20:4 and 22:6.

SM=sphingomyelin, CPG=choline phosphoglycerides, IPG + EPG=inositol plus serine phosphoglycerides, EPG=ethanolamine phosphoglycerides.

c. Hypoglycemia of 60 min duration. The results in Table 4 demonstrate that 60 min of hypoglycemia was associated with further changes in respiratory functions. First, although glutamate plus malate supported state 3 respiration showed little further reduction, the results indicate that succinate supported respiration was affected as well. Thus, in the presence of BSA plus KCl, state 3 respiration was reduced, with an accompanying reduction in RCR. Second, with succinate as substrate the ADP/O ratios showed a suggested decline. Third, although the recovery period was followed by normalization of state 3 respiration, it led to a rise in state 4 respiration, a change which was most apparent with glutamate plus malate as substrates, but probably also present with succinate as substrate (cf. RCR values). Fourth, the results strongly suggest that whatever the substrate used, recovery was associated with

Table 4. State 4 and state 3 respiratory activities (nmol O₂/mg mitochondrial protein⁻¹·min⁻¹), respiratory coefficient rates (RCR) and ADP/O ratios in rat cerebral mitochondria in controls (C), during hypoglycemia with an isoelectric EEG for 60 min (H) and after 90 min of recovery following hypoglycemia with an isoelectric EEG for 60 min (R).

Added substrate	State 4			State 3			RCR			ADP/O		
	C	H	R	C	H	R	C	H	R	C	H	R
<u>Malate+glutamate</u>												
+ MSTPI	14.5 +1.1	11.9 +1.1	21.1** +0.8	54.0 +2.9	26.1*** +1.8	63.3 +2.5	3.9 +0.2	2.2*** +0.0	3.1* +0.1	2.8 +0.1	2.7 +0.0	2.5** +0.1
+ MSTPI+2.5% BSA	13.0 +1.4	13.2 +1.0	20.6* +0.9	74.0 +2.6	36.9* +2.3	78.8 +2.3	5.9 +0.7	2.9* +0.2	3.9* +0.2	2.4 +0.1	2.5 +0.2	2.4 +0.1
+ MSTPI+100mM KCl	14.9 +1.4	13.5 +0.8	18.7 +0.8	62.3 +3.3	41.7* +1.0	75.6 +5.0	4.7 +0.1	3.2* +0.2	4.1* +0.1	2.5 +0.1	2.6 +0.1	2.4 +0.0
+ MSTPI+2.5% BSA + 100 mM KCl	10.4 +0.6	10.9 +0.9	15.3** +1.1	69.0 +3.5	46.7** +2.4	85.3 +6.2	6.8 +0.2	4.4*** +0.3	6.1 +0.6	2.6 +0.1	2.5 +0.0	2.4** +0.0
<u>Succinate</u>												
+ MSTPI	16.6 +0.7	17.0 +0.8	21.4** +1.1	57.0 +1.6	51.3 +1.8	58.7 +1.2	3.5 +0.2	3.1 +0.1	2.8** +0.1	1.8 +0.1	1.3* +0.0	1.5* +0.1
+ MSTPI+2.5% BSA	15.9 +1.0	18.5 +0.9	22.3* +1.2	67.1 +5.9	61.2 +2.1	64.1 +1.3	4.3 +0.5	3.4 +0.2	3.0 +0.2	1.6 +0.1	1.5 +0.0	1.6 +0.0
+ MSTPI+100mM KCl	15.1 +0.5	14.3 +0.8	17.0 +0.8	58.4 +1.8	56.6 +2.2	61.0 +1.3	3.9 +0.2	4.0 +0.1	3.7 +0.1	1.7 +0.0	1.5* +0.0	1.5** +0.0
+ MSTPI+2.5% BSA + 100mM KCl	13.9 +2.0	15.2 +0.9	17.2 +0.9	73.7 +2.2	60.6** +3.3	71.1 +3.8	5.3 +0.3	4.1** +0.2	4.2* +0.2	1.9 +0.1	1.6** +0.0	1.6*** +0.0

The values are means + S.E.M. * p<0.05; ** p<0.02; ***p<0.002 (Mann-Whitney U-test).

a moderate reduction in the ADP/O ratios.

3. Changes in cerebral energy state.

As discussed below, it is difficult to extrapolate results from in vitro measurements of mitochondrial respiratory function to the in vivo situation. We proceeded to measure, therefore, cerebral cortical concentrations of labile metabolites, reflecting cellular energy state, after 180 min of recovery following 60 min of hypoglycemia. Table 5 shows that as a result of a lingering reduction in ATP concentration, the adenylate energy charge was clearly subnormal; in addition, the lactate concentration was increased above normal. However, a comparison with results obtained after a recovery period of 30 min (data from Agardh et al. 1978, 1980) shows that cortical energy state improved in the period 30 to 180 min (see ATP and lactate values).

4. Changes in $[K^+]_e$ during recovery.

Since in vitro and in vivo results indicated that mitochondrial dysfunction was relatively moderate even after 60 min of hypoglycemia it seemed difficult to ascribe the failure of return of neurophysiological functions (Agardh and Rosén, in preparation) to loss of mitochondrial function. We, therefore, recorded extracellular K^+ activity after recovery periods corresponding to those employed for studies of mitochondrial function. As Fig.1 demonstrates, $[K^+]_e$ remained close to normal in the recovery period following 30 min of hypoglycemia. However, following 60 min of hypoglycemia $[K^+]_e$ rose steadily, reaching values of 8.0 ± 1.0 (Mean $\pm$ S.E.M.) to 17.3 ± 1.0 (mMol $\cdot$ l^{-1}) after 90 to 180 min of recovery.

In view of the fact that a small craniotomy had to be performed to allow measurements of $[K^+]_e$ we suspected that the procedure could have disturbed microcirculation and provoked an accentuation of any energy failure present. However, in the two experiments in which cortical tissue was frozen for metabolite analyses values obtained in the ipsilateral side did not differ from those of the contralateral side, nor did they differ from those illustrated in Table 5. We conclude, therefore, that loss of K^+ from cells reflect a metabolic lesion which helps to account for failure of functional recovery.

Table 5. Cerebral cortical concentrations of labile phosphates, lactate and pyruvate in the recovery period after insulin-induced hypoglycemia.

	Control	Isoelectric EEG 30 min and recovery (min)		Isoelectric EEG 60 min and recovery (min)	
		30 ^{a)} (n=4)	180 ^{a)} (n=4)	30 ^{b)} (n=7)	180 (n=6)
PCr	4.50 $\pm$ 0.06	4.97 $\pm$ 0.12 ^{xx}	5.48 $\pm$ 0.09 ^{xxx}	4.66 $\pm$ 0.11	5.06 $\pm$ 0.35
Cr	5.98 $\pm$ 0.17	5.36 $\pm$ 0.16	5.46 $\pm$ 0.24 ^x	4.89 $\pm$ 0.27 ^x	5.39 $\pm$ 0.56
ATP	2.95 $\pm$ 0.04	2.05 $\pm$ 0.09 ^{xxx}	2.39 $\pm$ 0.01 ^{xxx}	1.44 $\pm$ 0.03 ^{xxx}	1.89 $\pm$ 0.00 ^{xxx}
ADP	0.308 $\pm$ 0.011	0.297 $\pm$ 0.020	0.288 $\pm$ 0.017 ^x	0.271 $\pm$ 0.023	0.302 $\pm$ 0.020
AMP	0.038 $\pm$ 0.003	0.046 $\pm$ 0.009	0.032 $\pm$ 0.003	0.060 $\pm$ 0.005 ^{xx}	0.047 $\pm$ 0.005
Energy charge	0.942 $\pm$ 0.002	0.918 $\pm$ 0.005 ^{xx}	0.935 $\pm$ 0.002	0.889 $\pm$ 0.005 ^{xxx}	0.911 $\pm$ 0.005 ^{xxx}
Lactate	1.54 $\pm$ 0.07	2.66 $\pm$ 0.49	1.91 $\pm$ 0.15 ^x	6.38 $\pm$ 0.74 ^{xxx}	3.17 $\pm$ 0.49 ^{xx}
Pyruvate	0.109 $\pm$ 0.005	0.161 $\pm$ 0.020	0.127 $\pm$ 0.008	0.290 $\pm$ 0.014 ^{xxx}	0.186 $\pm$ 0.013 ^{xxx}

The values are given in $\mu\text{mol} \cdot \text{g}^{-1}$ wet weight and represent means $\pm$ S.E.M. x = $p < 0.05$; xx = $p < 0.01$; xxx = $p < 0.001$ (Student's t-test). a) Data from Agardh et al 1978, b) Data from Agardh et al 1980

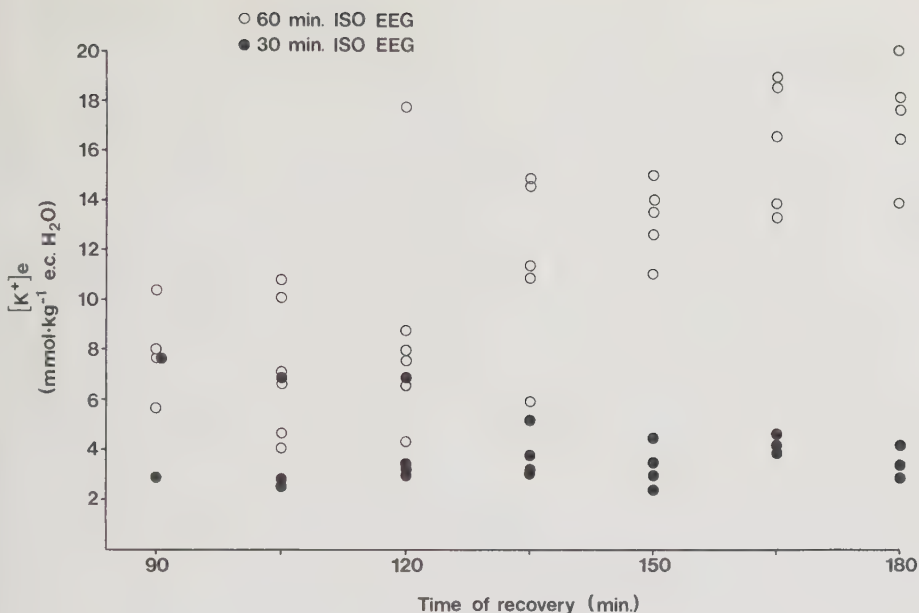


FIG.1. Cerebral cortical extracellular $[K^+]_e$ in the recovery period following hypoglycemia with an isoelectric EEG for 30 (●) and 60 (○) min.

DISCUSSION

When discussing the present results the following facts should be held in mind:

(a) Respiratory functions of isolated mitochondria reflect metabolic capacity rather than metabolic function since mitochondria are studied under optimal and artificial conditions remote from those prevailing in vivo. However, isolation of mitochondria also reduces their viability, as evidenced by irreversible loss of function ('ageing') upon prolonged incubation (for documentation and further literature, see Chefurka 1966, Chefurka and Dumas 1966). Thus, on the one hand, mitochondria from metabolically damaged tissue may respire more optimally in vitro than in vivo and, on the other hand, their isolation may provoke adverse changes that are artefactual. In the present study we are mainly concerned with two

such changes, i.e. the accumulation of free fatty acids (FFAs) and the loss of K^+ from mitochondria during isolation.

FFAs are known to have a detrimental effect on mitochondrial function and the effects observed include inhibition of oxygen uptake with reduced RCR values (Pressman and Lardy 1956, Björntorp et al. 1964, Lochner et al. 1978), inhibition of adenine nucleotide translocase (Wojtczak 1976, Shrago 1978), and uncoupling of oxidative phosphorylation (Pressman and Lardy 1956, Lehninger and Remmert 1959, Björntorp et al. 1964, Vázquez-Colón et al. 1966, Wojtczak 1976, Chefurka 1966). Since the isolation procedure activates phospholipases, albumin, an avid binder of FFAs, is included in the isolation medium together with EDTA (or EGTA) which chelates Ca^{2+} , an important activator of phospholipases. Furthermore, if respiration is inhibited in vitro, or uncoupled from oxidative phosphorylation, addition of BSA to the incubation medium may reveal if the effects are caused by accumulation of FFAs.

Brain mitochondria are known to require 50 to 100 mM K^+ in the incubation medium for optimal respiratory activity (Ozawa et al. 1967a, Clark and Nicklas 1970, see also Nicklas et al. 1971), and it has been shown that brain mitochondria are much more prone to loss of K^+ through, for instance, washing, than are other types of mitochondria. Brief periods of ischemia, another situation associated with elevated free fatty acid levels in the brain, also produce a rapid and extensive loss of K^+ in a low energy state (see Wojtczak 1976). Whatever the explanation, it seemed essential to study whether a reduction in respiratory function could be ameliorated by addition of K^+ especially since mitochondria are exposed to high K^+ activities in vivo.

(b) At first sight, studies of isolated mitochondria would appear to provide a more adequate approach to adverse reactions of importance for the development of cell damage than that provided by studies of cortical tissue in bulk. However, two facts indicate that this is not necessarily so. First, mitochondria derived from the whole supratentorial part of the brain include those of subcortical structures showing little evidence of neuronal cell damage. Second, at least in its un-

modified form, the isolation procedure of Clark and Nicklas (1970) seems to yield mitochondria of primarily glial origin (see Walsh and Clark, 1976). Since we lack information on the proportion of mitochondria of neuronal origin in our preparations some caution should be exercised when inferences are drawn from the present data.

With this as a background, we can proceed to discuss the present results. It seems profitable to do this by comparing them with those obtained in severe ischemia, a condition also involving loss of neuronal function and deterioration of cerebral energy state (for an account of mitochondrial changes in ischemia, see Schutz et al. 1973, Ikrényi et al. 1976, Ginsberg et al. 1977, Rehnrcrona et al. 1979).

1. Hypoglycemia of 30 min duration.

Like ischemia of 30 min duration (Rehnrcrona et al. 1979) hypoglycemia caused a reversible reduction of state 3 respiration. Since this occurred without any change in state 4 respiration, or in the ADP/O ratios, it reflects inhibition of respiration, and not uncoupling. Most, but not all, of this reduction in state 3 respiration (and RCR) could be reversed by addition of BSA and KCl, indicating that accumulation of FFAs and/or loss of K^+ were responsible.

In contrast to ischemia, hypoglycemia failed to affect respiratory activities when succinate was used as substrate. Our data are consistent with the hypothesis of a FFA-induced inhibition of pyridine nucleotide-linked respiration. Possibly, this inhibition is secondary to FFA-induced efflux of K^+ . Thus, K^+ activation of succinate-linked respiration in brain mitochondria is attained at a lower K^+ concentration than is required for the glutamate plus malate stimulated respiration (Ozawa et al. 1967a) which may explain the failure to observe any inhibition of the succinate oxidation.

The results on phospholipid and fatty acid composition of the mitochondrial fraction are seemingly in accordance with the moderate changes in respiratory functions. Nonetheless, the lack of changes in phospholipid content and composition is surprising, since mitochondria contain phospholipase A_2 (Woelk and Porcellati 1973).

The present results corroborate our previous conclusion that hypoglycemia is unaccompanied by gross signs of lipid peroxidation. Previous studies have shown that mitochondrial lipids, with their high content of polyenoic fatty acids, are prone to peroxidative attack, provided that the appropriate initiators are at hand (Ottolenghi 1959, Schneider *et al.* 1964, Flohé and Zimmerman 1974). In the present study, the concentrations of polyenoic fatty acids and of polyenoic phospholipids (e.g. EPG) remained unchanged. We conclude, therefore, that conditions during hypoglycemia do not favour free radical formation and lipid peroxidation.

2. Hypoglycemia of 60 min duration

Following 60 min of hypoglycemia, two additional changes were seen. First, some inhibition appeared to be present also of succinate-linked state 3 respiration. Second, at least when KCl was added to the incubation solution some decrease in the ADP/O ratios occurred. In analogy with what has been discussed above, the first of these changes might have been due to further loss of K^+ from the mitochondria. The decrease in ADP/O ratio is more difficult to explain, especially since it occurred without any accompanying increase in state 4 respiration. Conceivably, energy-dependent uptake of K^+ , with or without 'futile cycling' (see Wojtczak 1976), was the cause of the reduced ADP/O ratios.

The most prominent changes, following 60 min of hypoglycemia, were the further deterioration of mitochondrial function in the recovery period, and the progressive increase in extracellular K^+ activity. The mitochondrial changes, which involved increased state 4 respiratory rates and reduced ADP/O ratios, suggested true uncoupling of oxidative phosphorylation. In view of the fact that changes involving only inhibition of respiration probably are fully reversible, it is conceivable that true mitochondrial damage occurred or 'matured' in the recovery period. We can only speculate that part of the mitochondrial population, one that is derived from neuronal elements, suffered even greater damage than that observed in the population as a whole.

It is clear from what has been discussed above that results obtained on isolated mitochondria cannot be readily extrapolated to the in vivo situation. We measured, therefore, cerebral cortical concentrations of labile metabolites that should reflect mitochondrial function in vivo. As the results showed (see Table 5 above), there was no indication of progressive energy failure in the recovery period. Thus, although ATP concentrations and calculated energy charge values were reduced at 180 min (with an increased lactate concentration), a comparison with values obtained after 30 min of recovery indicated progressive improvement of cortical energy state thereafter.

The question arises whether the relatively moderate failure of mitochondrial function revealed by the in vitro and the in vivo data can explain the progressive rise in $[K^+]_e$ following 60 min of hypoglycemia. Since K^+ balance must be a function of the leak currents carried by monovalent cations and by the efficiency of the membrane-bound ATPase, it is conceivable that an increased 'leakiness' could be responsible. However, this possibility is difficult to reconcile with the progressive improvement of energy state. A second possibility is that the low ATP concentration and the increased concentration of P_i (which was not measured) reduces the free energy of ATP hydrolysis to values insufficient to achieve the coupled transport of K^+ and Na^+ (see Caldwell 1968). However, since there was a progressive improvement in the energy state and a significant increase ($p < 0.001$) in ATP concentration in the recovery period following 60 min of hypoglycemia, energy failure does not seem to explain the progressive rise in $[K^+]_e$.

We are then left with the possibility that 60 min of hypoglycemia causes damage to the membrane-bound ATPase. Thus, partial inactivation of this enzyme could explain why K^+ efflux does not measurably affect the phosphorylation state of the adenine nucleotide system. Further experiments are required to settle this point.

3. Relationship to other findings

It has already been pointed out that the loss of K^+ from cells provides a rational explanation for the failure of recovery of neurophysiological functions following 60 min of

hypoglycemia. Two further relationships emerge. First, the preservation of mitochondrial function following 30 min of hypoglycemia is in line with the absence of mitochondrial changes as observed by electron microscopy. Second, the deterioration of mitochondrial function during recovery following 60 min of hypoglycemia has its ultrastructural counterpart in the finding of increased electron lucency of their inner matrix (Kalimo et al. 1980)

4. Relationship to mitochondrial respiration in vivo.

At first sight, the results obtained after 30 min of hypoglycemia (inhibition of respiration without signs of uncoupling of oxidative phosphorylation) would seem to exclude the possibility that the disparity between CMR_{O_2} and cerebral energy state can be explained by uncoupling. However, such a conclusion is unwarranted in view of the fact that the isolation procedure optimizes conditions for respiratory activity (see above). In vivo, the mitochondria are exposed to increased levels of FFAs (see Agardh et al. 1981), and may suffer both uncoupling and loss of K^+ . Furthermore, conditions must favour intracellular accumulation of Ca^{2+} . This imposes a load on mitochondria to divert respiratory energy towards transport of ions and, since intracellular Na^+ concentration must rise, oxidative energy could be lost in energy requiring re-cycling of Ca^{2+} (see Crompton et al. 1978). Thus loose coupling due to accumulation of FFAs and futile cycling of K^+ and Ca^{2+} across the inner mitochondrial membrane could well explain why the cerebral energy state deteriorates in spite of an upheld CMR_{O_2} .

ACKNOWLEDGEMENTS

The authors are grateful to Kerstin Beirup and Lena Sjöberg for their excellent technical assistance, and to Gillian Sjö-dahl for typing the manuscript.

This study was supported by grants from Knut and Alice Wallenberg Foundation, the Swedish Medical Research Council (project No.14X-263) and from U.S. PHS (Grant No.2 R01 NS-07838).

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HYPOGLYCEMIC BRAIN INJURY. Metabolic and structural findings in rat cerebellar cortex during profound insulin-induced hypoglycemia and in the recovery period following glucose administration.

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ABSTRACT

Previous results have shown that severe, prolonged hypoglycemia leads to neuronal cell damage in, among other structures, the cerebral cortex and the hippocampus, but not in the cerebellum. In order to study whether or not this sparing of cerebellar cells is due to preservation of cerebellar energy stores, hypoglycemia of sufficient severity to abolish spontaneous EEG activity was induced for 30 and 60 min. At the end of these periods of hypoglycemia, as well as after a 30 min recovery period, cerebellar tissue was sampled for biochemical analyses or for histopathological analyses by means of light and electron microscopy.

After 30 min of hypoglycemia cerebellar energy state, defined in terms of the phosphocreatine, ATP, ADP, and AMP concentrations, was better preserved than in the cerebral cortex. After 60 min, though, gross deterioration of cerebellar energy state was observed in the majority of animals, and analyses of carbohydrate metabolites and amino acids demonstrated extensive consumption of endogenous substrates. In spite of this metabolic perturbation, histopathologic alterations were surprisingly discrete. After 30 min, no clear structural changes were observed and, after 60 min, only small neurons in the molecular layer (basket cells) were affected while Purkinje cells and granulae cells showed few signs of damage. The results support our previous conclusion that the pathogenesis of cell damage in hypoglycemia is different from that in hypoxia/ischemia, and indicate that other mechanisms than energy failure must contribute to neuronal cell damage in the brain.

INTRODUCTION

When insulin-induced hypoglycemia is of such severity that blood glucose concentrations are reduced to about or below $1 \mu\text{mol}\cdot\text{g}^{-1}$, spontaneous EEG activity ceases and extensive deterioration of cerebral energy state occurs (Hinzen and Müller 1971, Lewis et al. 1974b, Norberg and Siesjö 1976, Agardh et al. 1978). Due to lack of exogenous substrate, and to the maintained oxygen supply, hypoglycemia leads to extensive oxidative breakdown of endogenous carbohydrate and amino acid substrates (Cravioto et al. 1951, Goldberg, et al. 1966, Lewis et al. 1974a, Feise et al. 1976, Norberg and Siesjö 1976, Agardh et al. 1978), as well as of phospholipids and RNA (Hinzen et al. 1970, Agardh et al. 1981).

In man, severe and protracted hypoglycemia has been found to cause neuronal cell damage, mainly localized to the neocortex and the hippocampus (Meyer 1963, Brierley 1976). Since it remained unsettled whether complicating hypotension and/or hypoxia could have contributed to such damage, histopathologic studies were performed in animal experiments, in which systemic variables could be controlled. These studies verified that "ischemic" cell damage occurred in these selectively vulnerable areas (Brierley et al. 1971, Myers and Kahn 1971). In one of the studies, lesions were found in baboons in which blood glucose concentrations fell below $1.2 \mu\text{mol}\cdot\text{g}^{-1}$, and somatosensory evoked potentials were abolished for 49-92 min (Brierley et al. 1971). From the light microscopical picture observed, it was concluded that cell damage was similar in distribution and type to that observed in hypoxia/ischemia.

In the rat model of hypoglycemic "coma", it has been clearly shown that cell damage is unrelated to cerebral oxygen lack. Thus, cerebral blood flow (CBF) and oxygen availability has been found to be increased during severe hypoglycemia (Norberg and Siesjö 1976, Abdul-Rahman et al. 1980, Nilsson et al. 1981) and cellular redox systems to be oxidized (Lewis et al. 1974a). Recent studies of light microscopical and ultrastructural changes in the cerebral cortex have defined two types of neuronal damage, and indicated that the pathogenesis of damage is different from that in hypoxia/ischemia (Agardh et al. 1980,

Kalimo et al. 1980). Type I injury, characterized by angulated, darkly stained neurons with vacuolation of the perineuronal astrocytic processes, seemed to be irreversible in its severe form. Type II injury, characterized by moderate swelling of the neurons with subplasmalemmal clearing due to disintegration of ribosomes and reduction in the amount of rough endoplasmic reticulum (RER), appeared to represent a reversible form of injury.

The pathogenesis of hypoglycemic neuronal damage remains to be elucidated. Two possible mechanisms must be considered: deterioration of cerebral energy state, and oxidative breakdown of structural components. In the present study, we correlated changes in labile organic phosphates with histopathologic alterations in the cerebellum, a structure which seems largely resistant to hypoglycemic cell injury (Brierley et al. 1971, Brierley 1976). The objective of the study was to find out whether or not this resistance is related to preservation of cerebellar energy stores, e.g. due to a maintained glucose supply (cf. Abdul-Rahman and Siesjö 1980). Since a preserved glucose supply would curtail oxidation of endogenous substrate, we measured the major carbohydrate and amino acid substrates as well.

MATERIALS AND METHODS

Chemicals. Substrates and coenzymes for fluorometric analyses were purchased from Sigma (St. Louis), enzymes were from Boehringer and Sons (Mannheim) except lactate dehydrogenase which was from Millipore Corp. (New Jersey). Glutaraldehyde was obtained from Polaran Equipment (Watford, England). All other chemicals were analytical grade.

Animals, experimental series and groups. Male Wistar rats (270-420 g) of a S.P.F. Wistar strain (Møllegaard Avlslaboratorium, Copenhagen) were fasted over night before the experiments but had free access to tap water. The present communication describes the results of two series of animals. In series A, the animals were frozen in situ at the end of the experiments for later analysis of cerebellar (cortical) metabolites. In series B, the animals were fixed by perfusion for morpholog-

ical analyses with light and electron microscopy. The latter series is identical with a previous one in which structural alterations in the cerebral cortex were studied (Agardh et al. 1980, Kalimo et al. 1980). In order to allow a correlation of histopathological and metabolic alterations in the cerebellum, samples of cerebellar cortex were obtained from animals of that series and processed for light and electron microscopy. Furthermore, a new series of animals were prepared to obtain metabolic data, care being taken to match this material to the previous one.

Within each series, control animals injected with Krebs-Hens-leit solution were compared with animals rendered hypoglycemic by injection of an equal volume of insulin ($40 \text{ IU} \cdot \text{kg}^{-1}$ i.p. of Insulin NOVO Actrapid^(R)). Brains were sampled for biochemical analyses or for morphology when spontaneous EEG activity had ceased for either 30 or 60 min. In separate groups, recovery was induced for 30 min by infusion of glucose at the end of these periods of "isoelectric EEG" (rapid infusion of 0.5 ml of a 50% (w/v) glucose solution followed by a slow infusion for 30 min at a rate of $1 \text{ ml} \cdot \text{h}^{-1}$). A summary of the groups studied is given in Table 1 (see below). In each control group, the periods of anesthesia were matched to conform with those of the experimental series.

Operative and sampling techniques. About 60 min after insulin injection anesthesia was induced with 2-3% halothane; the animals were then tracheotomized, immobilized with an i.v. injection of tubocurarine chloride ($0.5 \text{ mg} \cdot \text{kg}^{-1}$) and ventilated with a Starling type respirator, delivering 70% N_2O and 30% O_2 , to yield an arterial P_{CO_2} of 30-40 mm Hg. Body temperature was maintained close to 37°C . One femoral artery was cannulated for continuous blood pressure recording with an electromanometer and for sampling of blood, and one femoral vein was cannulated for injections. The electroencephalogram (EEG) was continuously recorded in all animals from gold-plated copper bolts inserted into the skull bone in the fronto-parietal region (bipolar leads), using an Elema EEG machine.

After the operative procedures had been completed the animals were given Heparin i.v. (100 I.U.) and were maintained on 70% N_2O and 30% O_2 . Repeated arterial samples were anaerobically

taken from the catheter in glass capillaries for determination of pH, PCO_2 , and PO_2 or collected directly in liquid nitrogen for later analyses of glucose.

For sampling of cerebellar cortical tissue a skin incision was made over the skull bone to accommodate a plastic funnel for later freezing of the brain in situ with liquid nitrogen (Pontén et al. 1973).

Analytical techniques for biochemistry. The pH, PCO_2 and PO_2 in arterial blood were measured immediately after sampling, using microelectrodes operated at 37°C (Radiometer, Copenhagen, and Eschweiler & Co., Kiel) with appropriate temperature corrections. Blood and tissue samples were stored at -80°C until analysis. The brains were dissected and weighed at -20°C . Cerebellar cortical tissue was extracted at this temperature as described previously (Folbergrová et al. 1972a). Metabolites were determined with the fluorometric techniques of Lowry and Passonneau (1972). Analytical conditions have been previously described (Folbergrová et al. 1972a,b, 1974a,b).

Procedures for morphological analysis. Similar operative techniques were used as in series A. As described in the previous publications (Agardh et al. 1980, Kalimo et al. 1980), the sampling techniques and histopathological procedures were as follows. At the termination of the experiment a quick thoracotomy was performed, and a cannula was inserted in the ascending aorta via the left ventricle. Following a quick rinse with saline, 300 ml of 3% glutaraldehyde in $0.1 \text{ mol} \cdot \text{l}^{-1}$ phosphate buffer at pH 7.4 and 37°C as well as at a pressure of 135 mm Hg was perfused through the vascular bed. After perfusion the brain was allowed to stabilize in situ for 1-2 hours after which it was removed and stored in the same fixative until further processed. A piece of tissue was sampled from the upper part of the cerebellum and sectioned with a tissue sectioner (The Michle Lab. Ing., Gomshall, Surrey, England) in the sagittal plane to get approximately 500 μm thick sections from the vermis and the paramedian hemisphere of the cerebellum (Fig. 1). The sections were postfixed in 2% osmium tetroxide in cacodylate buffer, en bloc stained with 2% uranyl acetate in maleate buffer, dehydrated in a graded series of ethanol and embedded in epon. Large semithin sections were stained with toluidine blue for light microscopy.



Fig. 1. Samples for embedding in epon and subsequent light and electron microscopic analysis were taken from the stippled area of the cerebellar vermis and lateral to it.

Thin sections from the appropriate areas were sectioned with a LKB1 or Porter-Blum MT-2 ultramicrotome, double-stained with uranyl acetate and lead citrate and examined in a JEM 100C electron microscope.

Calculations and statistics. The "energy state" of the tissue was calculated in terms of the adenylate energy charge (E.C.) according to Atkinson (1968). Statistical differences were evaluated with Student's t-test. The following symbols are used: $x = p < 0.05$, $xx = p < 0.01$, $xxx = p < 0.001$.

RESULTS

As already mentioned, the present histopathologic results were obtained on cerebellar tissue sampled from a previous material in which neuronal changes in cerebral cortex were assessed (Agardh et al. 1980, Kalimo et al. 1980), while new groups for biochemical analysis were prepared to match those used for histopathology. Table 1 summarizes information on the various groups, and gives physiological parameters as well as blood glucose concentrations. Physiological variables were sufficiently similar in the biochemistry and morphology groups to exclude the possibility that differences in blood pressure, blood gases or pH could have influenced the conclusions drawn. During hypoglycemia, blood glucose concentrations were similarly reduced in the two series. Body temperature was close to 37°C in all animals (data not shown). Changes in EEG activity

Table 1. Physiological parameters and arterial blood glucose concentrations during insulin-induced hypoglycemia and in the posthypoglycemic recovery period.

Experimental group	Isoelectric EEG (min)	Recovery (min)	No. of animals	MABP (mmHg)	PaO ₂ (mmHg)	PaCO ₂ (mmHg)	pH	Glucose ($\mu\text{mol} \cdot \text{g}^{-1}$)
<u>BIOCHEMISTRY</u>								
A:1 (control)	-	-	16	156 $\pm$ 4	108 $\pm$ 2	37.7 $\pm$ 1.0	7.35 $\pm$ 0.13	6.86 $\pm$ 0.49
A:2	30	-	6	136 $\pm$ 6	102 $\pm$ 3	32.0 $\pm$ 1.0	7.37 $\pm$ 0.02	0.60 $\pm$ 0.08
A:3	60	-	7	127 $\pm$ 4	104 $\pm$ 7	35.7 $\pm$ 2.1	7.31 $\pm$ 0.15	0.56 $\pm$ 0.04
A:4	30	30	6	148 $\pm$ 3	108 $\pm$ 3	30.1 $\pm$ 1.2	7.27 $\pm$ 0.03	7.30 $\pm$ 0.58
A:5	60	30	7	146 $\pm$ 7	115 $\pm$ 5	34.4 $\pm$ 1.8	7.28 $\pm$ 0.03	7.94 $\pm$ 0.76
<u>MORPHOLOGY</u>								
B:1 (control)	-	-	5	147 $\pm$ 5	108 $\pm$ 6	35.7 $\pm$ 1.4	7.35 $\pm$ 0.01	5.76 $\pm$ 0.90
B:2	30	-	5	171 $\pm$ 11	117 $\pm$ 5	34.8 $\pm$ 2.8	7.35 $\pm$ 0.02	0.46 $\pm$ 0.08
B:3	60	-	3	118 $\pm$ 4	97 $\pm$ 6	39.8 $\pm$ 0.6	7.24 $\pm$ 0.02	0.56 $\pm$ 0.09
B:4	30	30	5	159 $\pm$ 8	113 $\pm$ 7	36.3 $\pm$ 1.9	7.24 $\pm$ 0.02	5.61 $\pm$ 0.58
B:5	60	30	3	148 $\pm$ 9	100 $\pm$ 6	34.2 $\pm$ 2.0	7.22 $\pm$ 0.02	9.07 $\pm$ 1.96

The values are means $\pm$ S.E.M. MABP = mean arterial blood pressure

during and following hypoglycemia were as described previously (see Lewis et al. 1974b). In no single animal was it possible to detect spontaneous EEG activity during the "isoelectric" periods (30 or 60 min).

1. Biochemistry

Changes in biochemical variables are shown in Table 2. Hypoglycemic coma of 30 min duration gave rise to a relatively extensive deterioration of cerebral energy state, which was further accentuated after 60 min. Thus, the concentrations of PCr and ATP were reduced, and those of creatine, ADP, and AMP increased. As a result, the calculated adenylate energy charge decreased substantially. The sum of adenine nucleotides (ATP+ADP+AMP) was reduced, indicating degradation of AMP via the AMP deaminase (E.C. 3.5.4.17) and/or 5'-nucleotidase (E.C. 3.1.3.31) reactions.

The results of Table 2 indicate a considerable variability in results between animals. Fig. 2 gives individual values for adenine nucleotides and energy charge, related to blood glucose concentrations. The data illustrate three points. First, in the 30 min group two of six animals had values that were just outside the normal range while the remainder showed extensive deterioration of tissue energy state. Second, in the 60 min group one of seven animals had a surprisingly moderate perturbation of energy state while the results of the remaining six were consistent. Third, the metabolic perturbation showed no obvious relationship to glucose concentration in blood (or tissue, data not shown).

Changes in substrate levels (see Table 2) demonstrated utilization of both carbohydrate and amino acid substrates. Thus, glycogen and glucose were reduced to low levels and there were highly significant decreases in lactate and pyruvate concentrations. As previously observed in cerebral cortex (Norberg and Siesjö 1976, Agardh et al. 1978) changes in amino acids were those expected from substrate utilization by means of transamination (reduction in glutamate and increase in aspartate concentrations) and deamination (reduction in amino acid pool size).

Table 2. Cerebellar cortical concentrations of labile phosphates, carbohydrate substrates and amino acids during hypoglycemia and in the recovery period following glucose injection.

	Control (n=16)	Hypoglycemia with isoelectric EEG(min)		Recovery 30 min following iso- electric EEG (min)	
		30 (n=6)	60 (n=7)	30 (n=6)	60 (n=7)
ATP	2.65 $\pm$ 0.03	1.51 $\pm$ 0.27***	1.04 $\pm$ 0.23***	2.29 $\pm$ 0.15**	1.80 $\pm$ 0.12***
ADP	0.252 $\pm$ 0.007	0.576 $\pm$ 0.087***	0.665 $\pm$ 0.068***	0.249 $\pm$ 0.017	0.296 $\pm$ 0.022*
AMP	0.043 $\pm$ 0.002	0.228 $\pm$ 0.058***	0.230 $\pm$ 0.041***	0.052 $\pm$ 0.008	0.073 $\pm$ 0.010***
Energy charge	0.942 $\pm$ 0.001	0.763 $\pm$ 0.052***	0.694 $\pm$ 0.039***	0.942 $\pm$ 0.004	0.898 $\pm$ 0.006***
PCr	6.15 $\pm$ 0.06	2.98 $\pm$ 0.91***	1.75 $\pm$ 0.71***	6.91 $\pm$ 0.09***	6.87 $\pm$ 0.16***
Cr	7.63 $\pm$ 0.20	10.74 $\pm$ 0.76***	11.31 $\pm$ 0.73***	6.61 $\pm$ 0.28**	6.94 $\pm$ 0.29*
Glycogen	3.50 $\pm$ 0.08	0.28 $\pm$ 0.01***	0.23 $\pm$ 0.09***	0.59 $\pm$ 0.12***	0.46 $\pm$ 0.12***
Glucose	3.36 $\pm$ 0.02	0.16 $\pm$ 0.02***	0.20 $\pm$ 0.04***	4.17 $\pm$ 0.50*	4.77 $\pm$ 0.41***
Lactate	0.78 $\pm$ 0.06	0.52 $\pm$ 0.06*	0.37 $\pm$ 0.06***	1.32 $\pm$ 0.06***	1.77 $\pm$ 0.17***
Pyruvate	0.07 $\pm$ 0.00	0.03 $\pm$ 0.01***	0.04 $\pm$ 0.00***	0.08 $\pm$ 0.00	0.12 $\pm$ 0.01***
Glutamate	10.11 $\pm$ 0.23	5.82 $\pm$ 1.46***	3.03 $\pm$ 1.02***	10.52 $\pm$ 0.20	9.14 $\pm$ 0.22*
Aspartate	2.87 $\pm$ 0.09	9.17 $\pm$ 1.88***	9.27 $\pm$ 0.11***	2.62 $\pm$ 0.12	1.93 $\pm$ 0.10***
Glutamine	5.54 $\pm$ 0.19	2.84 $\pm$ 0.69***	1.35 $\pm$ 0.37***	4.50 $\pm$ 0.54*	2.18 $\pm$ 0.44***
Alanine	0.302 $\pm$ 0.08	0.288 $\pm$ 0.043	0.224 $\pm$ 0.37**	0.458 $\pm$ 0.034***	0.468 $\pm$ 0.012***
GABA	2.05 $\pm$ 0.17	1.01 $\pm$ 0.12**	1.45 $\pm$ 0.16*	1.72 $\pm$ 0.05	2.51 $\pm$ 0.23

The values are given in $\mu\text{mol}\cdot\text{g}^{-1}$ wet weight and represent means $\pm$ S.E.M.

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

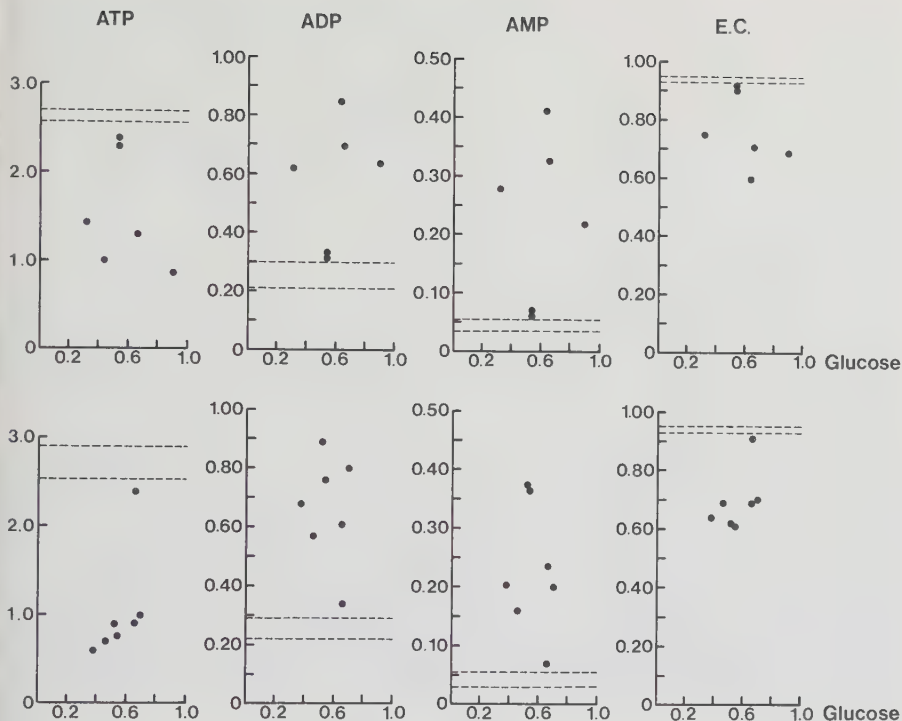


Fig. 2. Individual cerebellar cortical concentrations of ATP, ADP, AMP and energy charge (E.C. ordinate) related to arterial blood glucose (abscissa) after 30 (top) and 60 (bottom) min of isoelectric EEG. The interrupted lines indicate the ranges of the control values. The values are given in $\mu\text{mol}\cdot\text{g}^{-1}$ wet weight.

Following 30 min of hypoglycemic coma, recovery of cerebral energy state showed three distinct features: an increase above normal in the PCr/creatine ratio, a lingering reduction in adenine nucleotide pool size, and a return of energy charge to normal. After 60 min of coma, the size of the adenine nucleotide pool was further reduced and significant increase in ADP and AMP concentrations were observed. As a result the adenylate energy charge failed to normalize. Changes in the lactate/pyruvate couple also suggest a lingering perturbation of cerebellar energy metabolism. Thus, in both groups, posthypoglycemic lac-

tic and pyruvic acid concentrations rose above control, and the lactate/pyruvate ratio was elevated (data not shown).

After 30 min of hypoglycemic coma, extensive restoration of amino acid concentrations occurred. After 60 min of coma, though, only the GABA concentration returned to normal and, at the end of the 30 min recovery period, the amino acid pool size was still decreased by about $5 \mu\text{mol}\cdot\text{g}^{-1}$.

2. Histopathology

a. Hypoglycemia. In control animals, both the light (LM) and electron microscopic (EM) structure of the cerebellar cortex corresponded to that generally considered normal (Palay and Chan-Palay 1974).

Considering the biochemical perturbation induced by hypoglycemia, the structural alterations were surprisingly discrete. At the end of a 30 min period of ceased EEG activity no alterations from the normal could be observed in LM. Analyses by EM gave similarly negative findings, as exemplified by the normal appearance of Purkinje cells and granule cells in Fig. 3.

Even after 60 min of hypoglycemic coma very few changes were seen, indicative of cell damage. In two of the three animals a few small, angulated, darkly stained cells were observed in the molecular layer over the crests of a few of the folia in LM. These were similar to the type 1 injured neurons in the cerebral cortex (Agardh et al. 1980, Kalimo et al. 1980). Furthermore, at one location we observed one single, condensed and microvacuolated larger neuron (probably a Purkinje cell) and one slightly condensed cell, probably a Golgi cell (Fig. 4). However, in LM other Purkinje cells appeared normal and the only consistent alteration observed was a suggested swelling of basket cells (Fig. 5). EM confirmed the absence of clear changes from the normal in Purkinje cell morphology (Fig. 6). Thus, the nuclear chromatin was evenly distributed and the cytoplasmic organelles were strikingly intact. It appeared, though, that the mitochondrial matrix was slightly condensed ("contraction of mitochondria"). Furthermore, in some Purkinje cells the number of RER cisternae appeared to be decreased and the remaining cisternae to be relatively short and haphazardly oriented (not shown).

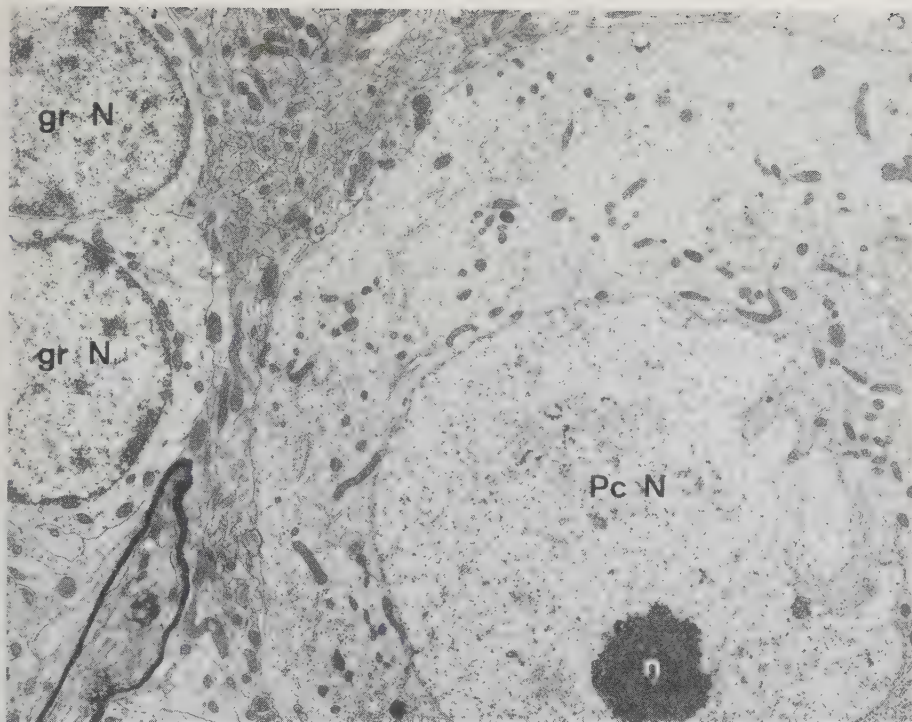


Fig. 3. No essential ultrastructural change is seen in the Purkinje (Pc) or granule (gr) cells after 30 min of isoelectric hypoglycemia. The indentation of the nuclear envelope in the Purkinje cell is a normal feature as is also the condensation of the chromatin in the granule cell nuclei. The mitochondria appear quite compact, RER is normally distributed and ribosomes form rosettes. The cell processes and their organelles in the neuropil appear normal. N = nucleus, n = nucleolus. 6000 x.

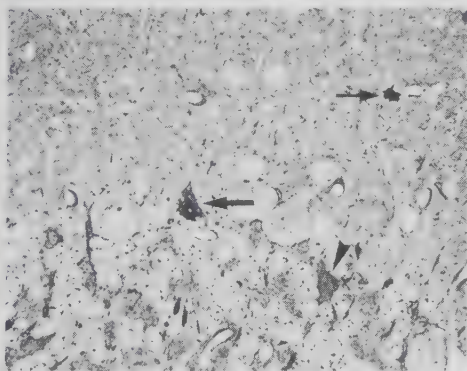


Fig. 4. In one animal exposed to 60 min of hypoglycemia in the cortex over the crest of a folium a focus with a darkly stained microvacuolated probable Purkinje cell (thick arrow) and a slightly condensed probable Golgi cell (arrowhead) were seen. These were the only ones found and we did not succeed to obtain them for EM. In the molecular layer there is also a darkly stained, small neuron (thin arrow, for explanation see text). 345 x.

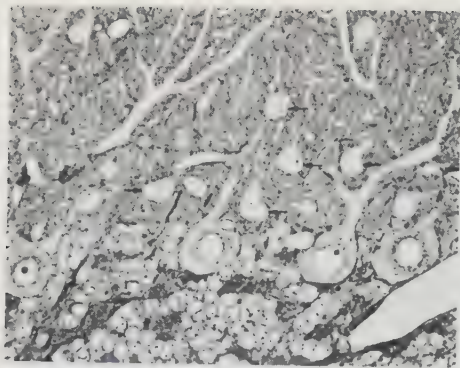


Fig. 5. After 60 min of hypoglycemia the only constant light microscopic change is the slight swelling of the basket cells (arrows) with occasional tiny vacuoles in their cytoplasm. The neuropil in the molecular layer displays some vacuoles which obviously represent the swollen dendrites of the basket cells. The large Purkinje cells as well as the tightly packed granule cells beneath them appear normal. Epon + toluidine blue. 345 x.

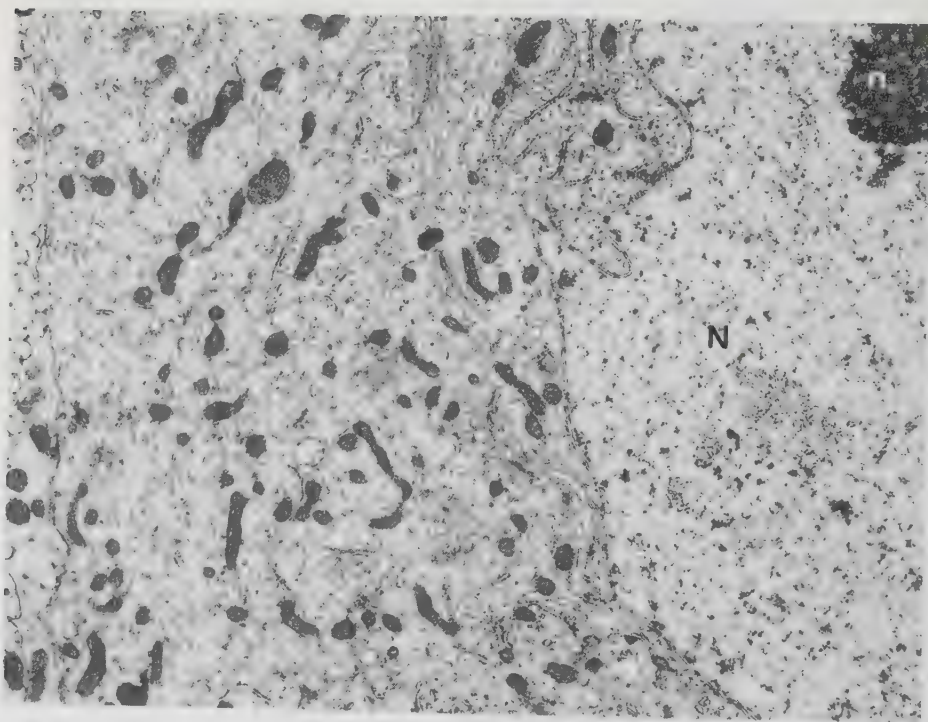


Fig. 6. After 60 min of hypoglycemia even the ultrastructural changes in the Purkinje cells were minimal. Nuclear chromatin is evenly distributed. Ribosomes form rosettes, RER and Golgi cisternae appear normal in shape and distribution and mitochondria are condensed. N = nucleus, n = nucleolus. 10 600 x.

After 60 min of hypoglycemia also the granule cells appeared normal except for a suggested contraction of their mitochondria and, possibly, a somewhat increased electron lucency of their cytoplasm (Fig. 7). Their nuclear chromatin was condensed as it is normally. Synaptic structures in all three cortical layers and most dendritic and axonal processes (see below) appeared unaltered (see Fig. 3 above).

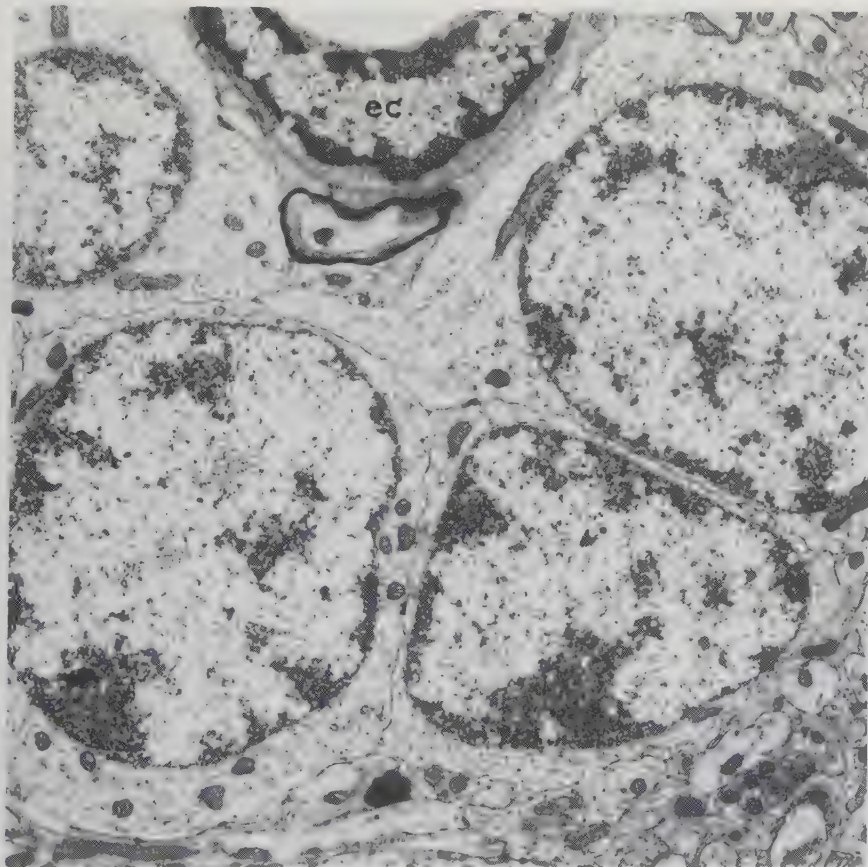


Fig. 7. After 60 min of hypoglycemia the granule cells likewise show only minimal changes. Mitochondria appear slightly condensed and the cytoplasm is somewhat more electron lucent than normally. The nuclear chromatin is condensed in peripheral blocks, but this is the normal distribution in granule cells. ec=capillary endothelial cell. 10 000 x.

The only nerve cells that showed a clearly adverse reaction to the hypoglycemic insult were the small neurons (mostly basket cells) in the molecular layer. In epon sections these cells appeared somewhat swollen with pale cytoplasm, in which small clear vacuoles were often visible (see Fig. 5 above). In EM (Fig. 8) their mitochondria were clearly distended ("high amplitude swelling") with disruption of the cristae. Furthermore, the cell sap was watery and flocculent, the Golgi cisternae were dilated and membranous whorls were commonly seen in the cytoplasm. In the neuropil of the molecular layer many small dendrites (obviously originated from the basket cells described) were similarly swollen and many of them contained damaged mitochondria.

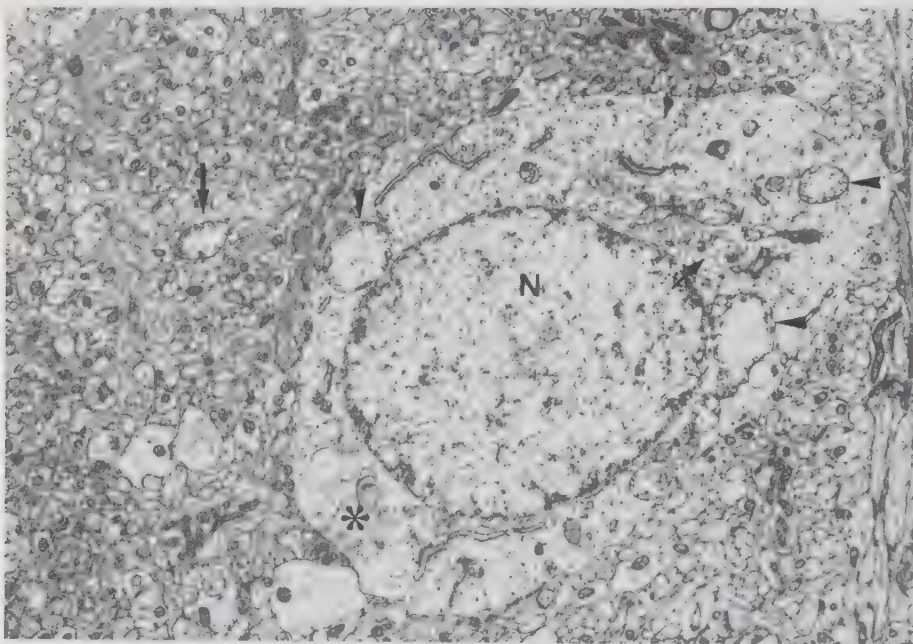


Fig. 8. After 60 min of hypoglycemia mitochondria (arrowheads) in a basket cell are markedly swollen with disrupted cristae. The cytoplasm displays somewhat increased electron lucency. Nuclear (N) chromatin is very slightly coarser than normal. In the surrounding, quite compact neuropil one process, most probably of basket cell origin, contains a similarly swollen mitochondrion (thick arrow). Golgi cisternae (thin arrow) are slightly swollen. A membraneous whorl is seen in the cytoplasm (asterisk). 6 900 x.

The Fañanas astrocytes in the vicinity of the Purkinje cells often became more prominent during the 60 min period of hypoglycemia: the nuclei appeared slightly enlarged and distended, but with finely dispersed chromatin. Sometimes their cytoplasm appeared watery but extensive swelling as often seen in ischemic injury was not observed. In EM one could observe the characteristic finely granular chromatin and the gathering of their fairly intact organelles at one pole of the cytoplasm (Fig. 9). Remarkably, no dilatation of the perivascular astrocytic processes was seen.

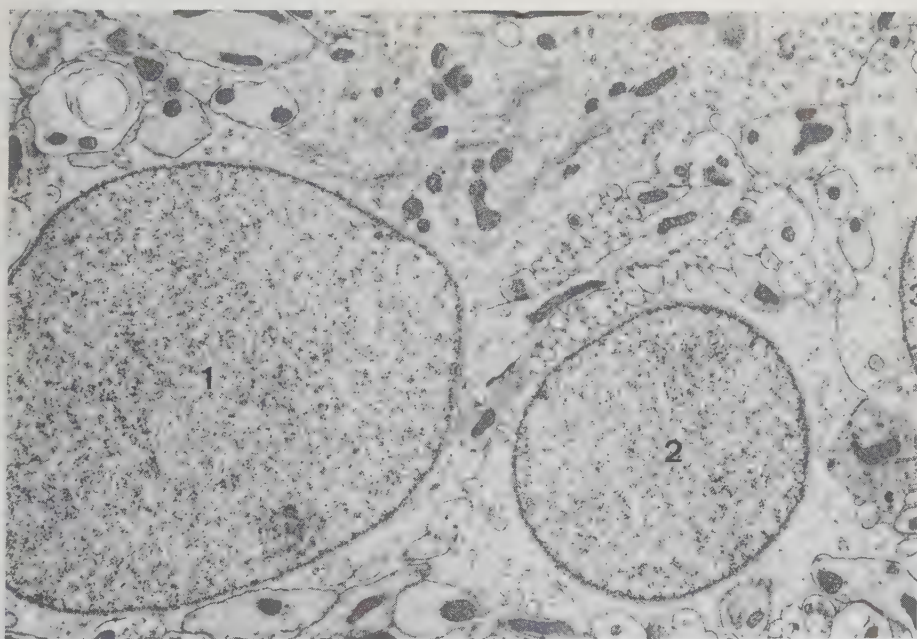


Fig. 9. Three Fañanas astrocytes after 60 min of hypoglycemia again with minimal changes. Chromatin is evenly distributed in the rounded, slightly distended nuclei. Cytoplasm in cells 2 and 3 contains very few organelles, but in none of the cells does it appear edematous. In cell 1 the mitochondria appear condensed, short RER cisternae are rounded, but Golgi cisternae are narrow, elongated. 6 400 x.

b. Recovery. Since changes during hypoglycemia were slight, one could expect alterations during recovery to be even less conspicuous (cf. Agardh et al. 1980, Kalimo et al. 1980). Since no clear alterations from control were observed after 30 min of hypoglycemia, the description is confined to the 60 min group. The Purkinje cells appeared normal (Fig. 10). Possibly, the mitochondria were slightly less contracted after the recovery than during hypoglycemia but no signs of mitochondrial damage were observed. In a few cells, the ribosomal rosettes were less regular than in the controls and the amount of RER seemed slightly reduced. However, since no morphometrical analysis was performed definite conclusions cannot be made.

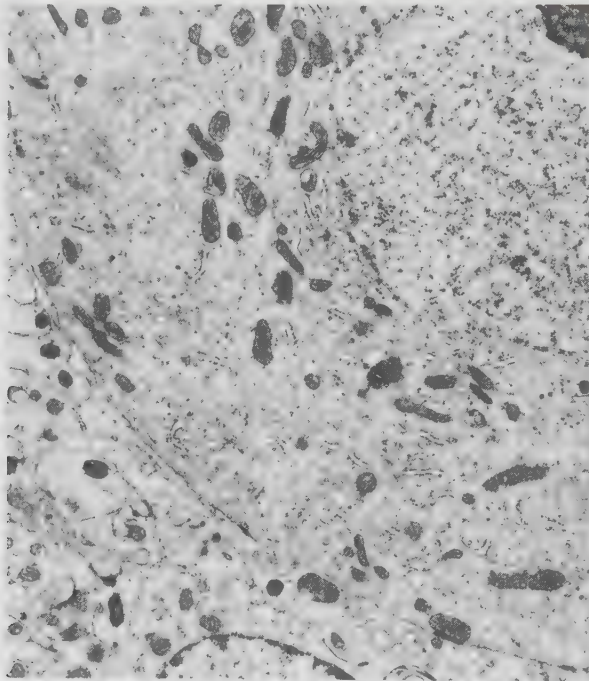


Fig. 10. After 60 min of hypoglycemia followed by 30 min recovery the Purkinje cell appears similar as immediately after the hypoglycemia. N = nucleus. 8 800 x.

The basket cells in the molecular layer still showed swollen mitochondria but no additional alterations were seen (Fig. 11). In the LM, the Fahnas astrocytes appeared less prominent, and EM showed that their mitochondria were slightly less contracted. Otherwise, no striking change had occurred during the re-stitution.

In one animal in the 60 + 30 min group a few darkly stained neurons similar to those described above were seen in the molecular layer over a crest of a folium.

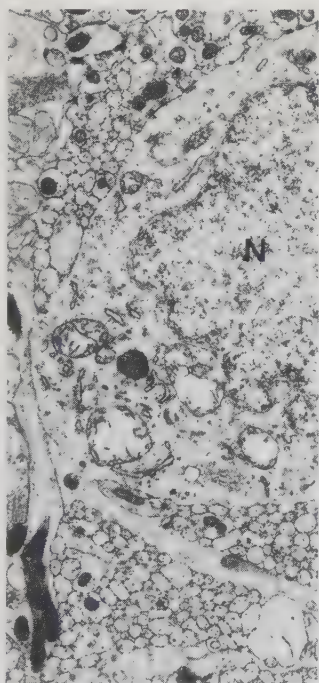


Fig. 11. The basket cell from the same animal as Fig. 10 likewise appears similar as during hypoglycemia (cf. Fig. 8). During the short recovery period neither further disintegration nor definite recovery became evident. N = nucleus. 7 850 x.

DISCUSSION

The present results demonstrate that although hypoglycemic "coma" leads to a relatively extensive energy failure in the cerebellum, especially after 60 min, histopathological alterations were surprisingly discrete, with virtually complete sparing of Purkinje cells. In view of the results reported by others (Meyer 1963, Brierley et al. 1971, Brierley 1976) the

absence of widespread cell damage was not surprising. However, what was unexpected was the fact that cell morphology was preserved in spite of a relatively extensive deterioration of cellular energy metabolism. Since this finding hints that depletion of energy reserves is not necessarily followed by cell damage it seems warranted to discuss in some more detail metabolic alterations in cerebral cortex and cerebellum.

1. Biochemical alterations in neocortex and cerebellum

Metabolic changes in the cerebral cortex (which shows extensive morphologic alterations, see Agardh et al. 1980, Kalimo et al. 1980) have been extensively documented in hypoglycemic coma of maximally 60 min duration (Lewis et al. 1974a,b, Feise et al. 1976, Norberg and Siesjö 1976, Agardh et al. 1978, 1980). These studies have shown that extensive deterioration of cortical energy state occurs pari passu with the cessation of spontaneous EEG activity, and that little further change occurs in the subsequent 60 min period. Thus, for the neocortex it is possible to define the duration of the deterioration in energy state corresponding to the structural alterations observed. For the cerebellum, this is not possible since metabolic measurements have not been performed at shorter "coma" duration than 30 min. Therefore, we cannot exclude the possibility that cell damage was absent in the 30 min group because the period of overt energy failure was less than 30 min. Accordingly, we have to lay some emphasis on the correlation between biochemical and morphological alterations in the 60 min group.

To facilitate discussion, we have compared percentage changes from control in labile metabolites reflecting cellular energy state in the neocortex (data from Agardh et al. 1978, 1980) and the cerebellum (present results). In this comparison (Fig. 12), the single aberrant animal in the 60 min group was excluded. The results show that, after 30 min, changes in tissue energy state were less marked in the cerebellum. It should be recalled, though, that 4 of 6 animals showed more extensive metabolic alterations than what Fig. 12 suggests. After 60 min, the changes between the two tissues were less marked. From these results we conclude two things. First, although some factors delay the development of extensive energy failure in

the cerebellum they cannot prevent such failure from occurring during prolonged hypoglycemic coma. Second, since all animals studied after 30 min had some deterioration of tissue energy state those studied after 60 min must have suffered a relatively extensive period of cerebellar energy failure for at least 30 min.

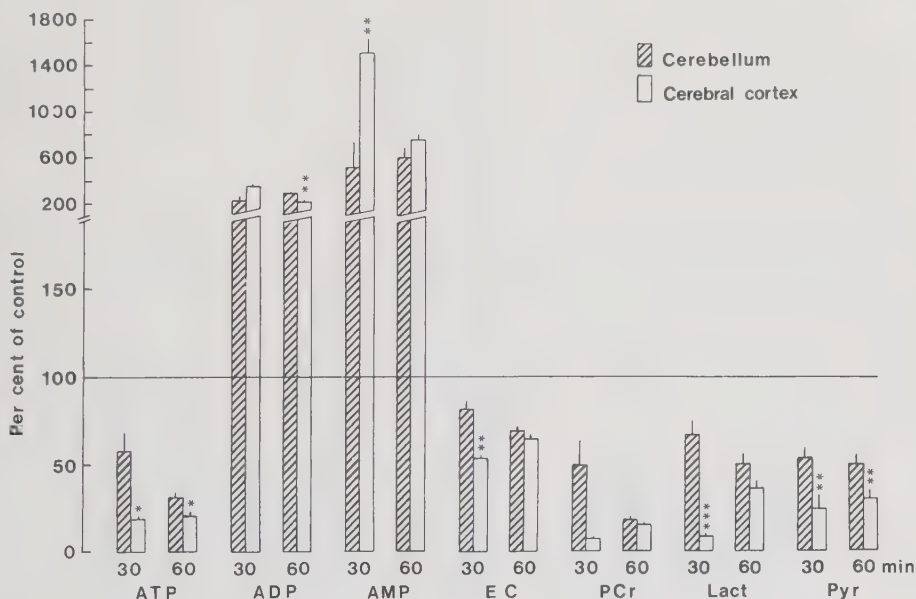


Fig. 12. Cerebellar and cerebral cortical concentrations of ATP, ADP, AMP, PCr, lactate, pyruvate and energy charge (E.C.) during hypoglycemia with an isoelectric EEG for 30 and 60 min. The values are given in per cent of control values for respective brain region and represent means $\pm$ S.E.M. Statistical differences are calculated between the experimental groups. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Fig. 13 illustrates the corresponding changes in amino acid substrates. The results demonstrate that, after 30 min, "consumption" of endogenous substrates was less in the cerebellum than in the neocortex. However, after 60 min the changes were similar, indicating that energy failure in the cerebellum (as in the neocortex) is due to restriction of glucose supply and

"exhaustion" of endogenous substrates. We conclude from these results that after 60 min of hypoglycemia, metabolic perturbation in the cerebellum was at least as pronounced as that observed in the neocortex after 30 min of hypoglycemia.

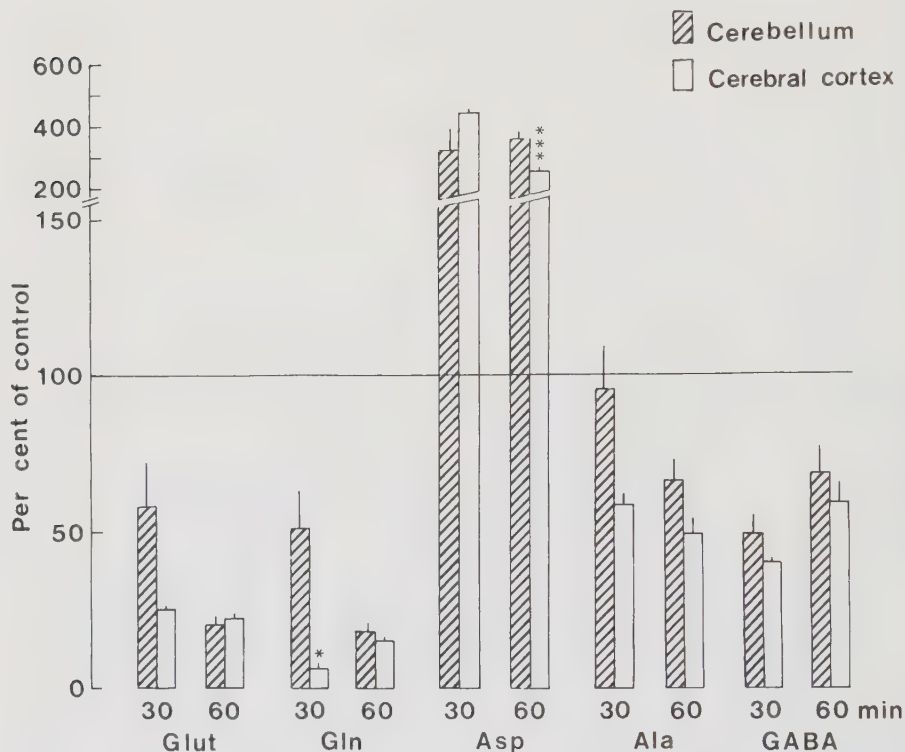


Fig. 13. Cerebellar and cerebral cortical concentrations of glutamate, glutamine, aspartate, alanine and GABA during hypoglycemia with an isoelectric EEG for 30 and 60 min. The values are given in per cent of control values for respective brain region and represent means $\pm$ S.E.M. Statistical differences are calculated between the experimental groups. * $p < 0.05$, *** $p < 0.001$.

In the recovery period, restitution of metabolite levels was relatively similar in neocortex and cerebellum (data not shown). Like the neocortex the cerebellum showed incomplete recovery of cerebral energy state. For example, after 60 min of hypoglycemia the adenylate energy charge was clearly subnormal and

the lactate concentration as well as the lactate/pyruvate ratio were increased. Probably, these changes reflect metabolic damage incurred during the hypoglycemia. It should be emphasized, though, that we know nothing of the potential reversibility of these alterations.

2. Structural alterations

As remarked previously, hypoglycemic coma of 30 min duration leads to extensive structural alterations in the cerebral cortex and, although most cells appeared to recover following glucose administrations, about 2% showed signs of irreversible damage (Agardh et al. 1980, Kalimo et al. 1980). After a 60 min period of hypoglycemia, histopathologic damage was even more severe. In the cerebellum, no clear alterations were observed after 30 min and those seen after 60 min were strikingly discrete. Perhaps most importantly, the Purkinje cells were almost completely spared. The reduction of RER in some of the Purkinje cells after 60 min of hypoglycemia suggested an impending type II injury (in the cerebral cortex, this type mainly affected larger neurons in layers 5-6). However, definite clearing of the peripheral cytoplasm, disintegration of the ribosomes and elongation of RER cisternae, all characteristic of the type II injured neurons, were not present.

Since also the granulae cells appeared unaffected by the hypoglycemia, and since alterations of the Fañanas astrocytes were relatively slight, the only histopathologic changes suggestive of serious damage were those affecting the small neurons in the molecular layer, defined as basket cells. The alterations affecting these cells, i.e. the high amplitude swelling of the mitochondria, are neither seen in type I nor in type II injury in the cerebral cortex, but rather resembles the early, "microvacuolated" stage of nerve cell injury observed in transient hypoxia/ischemia (Brown and Brierley 1972, Garcia et al. 1977). Yet, no karyocytoplasmic condensation was seen in the basket cells.

3. Energy failure and cell damage

Neuronal cell damage with a selective localization to neocortex, hippocampus and some other brain structures has been observed in hypoxia/ischemia and in hypoglycemia, two condi-

tions associated with gross deterioration of cerebral energy state. It has been natural to assume that the damage incurred is related to the energy failure. However, our previous results have indicated that the histopathologic alterations in severe hypoglycemia are different from those previously described for hypoxia/ischemia. The present results demonstrate that, in spite of extensive energy failure most cerebellar cells, including the "vulnerable" Purkinje cells, fail to show signs of definitive damage. The results add further support to the conclusion that the pathogenesis of cell damage in hypoglycemia is different from that of hypoxia/ischemia. Furthermore, the results make it questionable that a simple relationship exists between energy failure and cell damage. Two pertinent questions must be posed. First, what factors are responsible for the vulnerability of certain cells to hypoxia/ischemia and to hypoglycemia, respectively? Second, if exhaustion of energy stores cannot adequately explain that cell damage is incurred, what are the additional mechanisms involved? Our continued work is directed towards these questions.

Acknowledgements

The skilful assistance of Kerstin Beirup, Karin Hansson, Gertie Johansson, Yvonne Hansson, Gillian Sjödahl, and Frank Bittkowski is gratefully acknowledged. This study was supported by grants from the Swedish Medical Research Council (No. 14X-263, 12X-0302), and from US PHS (No. R01 NS07838).

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HYPOGLYCEMIC BRAIN INJURY. Labile metabolites reflecting cellular energy state, cyclic nucleotides, and free fatty acids in the cerebellum of the rat after 30 and 60 min of severe insulin-induced hypoglycemia.

Carl-David Agardh and Bo K. Siesjö

ABSTRACT

Previous results from the laboratory have shown that although prolonged severe hypoglycemia (30 or 60 min with ceased EEG activity) leads to relatively extensive energy failure in the cerebellum, there is surprisingly little histopathologic evidence of cell damage, with virtually complete sparing of the Purkinje cells. In the present study, we induced hypoglycemia of such durations and compared metabolic alterations in the cerebral cortex (a structure which shows extensive cell damage) and the cerebellum. The experiments were performed in rats ventilated on 70 per cent N_2O and 30 per cent O_2 , and hypoglycemia was induced by insulin administration.

After 30 and 60 min of hypoglycemia the majority of animals showed changes in cerebellar energy state, considerably less pronounced than in the cerebral cortex. In some animals, useful amounts of glycogen remained even after 60 min of hypoglycemic coma. It is concluded that part of the resistance of the cerebellum in hypoglycemia is due to a better preserved substrate supply, probably since the cerebellum contains a higher density of glucose transport sites. However, this alone cannot explain the higher resistance of the tissue. Thus in all 30 min animals the cerebellum showed some deterioration of cellular energy state and, after 60 min the majority had relatively extensive energy failure. Furthermore, the results obtained in animals with a clearly reduced adenylate energy charge suggested that some decrease in phospholipid content occurred.

During hypoglycemia, the cerebellar concentrations of cAMP and cGMP rose. The concentration of cGMP, but not that of cAMP, showed an inverse relationship to the energy charge. In cerebellum, cGMP rose to higher values than in the cortex, and in contrast to cerebral cortical cAMP concentration that in the cerebellum showed no tendency to secondary reduction with progressive deterioration of tissue energy state.

During hypoglycemia, the concentrations of free fatty acids increased in the cerebellum, the values showing an inverse relationship to cellular energy state. However, the accumulation was less pronounced than in the cerebral cortex,

even when the two structures were compared at similar degrees of deterioration of energy state. Since accumulation of polyenoic FFA has been implicated in tissue damage in several adverse situations, the results hint that part of the resistance of the cerebellum to the hypoglycemic insult may be due to a better preserved Ca^{2+} homeostasis and/or a less pronounced activation of phospholipase A_2 .

INTRODUCTION

In a previous communication (Agardh et al. 1981a) we correlated changes in metabolites reflecting deterioration of cellular energy state and consumption of endogenous substrate stores to histopathology in the cerebellum of rats subjected to severe hypoglycemia of 30 and 60 min duration (with cessation of spontaneous EEG activity). Although metabolic changes had a more gradual onset in the cerebellum than in the cerebral cortex, 60 min of hypoglycemia was nevertheless associated with signs of gross metabolic failure. In spite of this, histopathologic damage was slight and the Purkinje cells were virtually unaffected. The results emphasized, therefore, that a poor relationship seems to exist between energy failure and cell damage.

In the present study, we have further explored mechanisms that may induce cell damage. As described in detail elsewhere (Siesjö 1981, see also Discussion) a common feature of three conditions leading to cell damage in the brain (hypoxia/ischemia, hypoglycemia, and status epilepticus) is the accumulation of free fatty acids (FFA), particularly arachidonic acid. There is evidence suggesting that oxidative metabolism of arachidonic acid, e.g. via the fatty acid cyclo-oxygenase and the lipoxxygenase pathways, can induce adverse cellular reactions. The primary objective of the present experiments, therefore, was to explore whether accumulation of FFA in the cerebellum is less pronounced than in the cerebral cortex and, if so, if this could be related to a less marked deterioration of cellular energy state. However, since evidence exists that some guanylate cyclase systems are activated by hydroperoxide breakdown products of arachidonic acid (Asano and Hidaka 1977, Murad et al. 1979) we measured the tissue contents of cyclic nucleotides as well.

MATERIALS AND METHODS

Chemicals. Substrates and coenzymes for fluorometric analyses were purchased from Sigma (St. Louis); enzymes were from Boehringer and Sons (Mannheim) except lactate dehydrogenase which was from Millipore Corp. (New Jersey). Cyclic nucleotides were measured using commercial kits: Cyclic AMP Assay kit, Radio-

chemical center, Amersham, England, and Cyclic GMP (^{125}I -labeled) RIA kit, New England Nuclear, Boston, U.S.A. Chloroform, tuolene, diethylether, light petroleum (b.p. $40^{\circ}\text{--}60^{\circ}\text{C}$) and siliaca gel-60 were from Merck, Darmstadt, W.Germany. Methanol Pronalys was from May and Baker Ltd., Dagenham, England. BHT (2,6-ditert.-butyl-p-cresol) was from Fluka AG, Buchs, Switzerland and Heneicosanoic acid (21:0) from Larodan Lipids AB, Malmö, Sweden. Standards of fatty acid methyl esters were from Serdary Research Laboratories, Ontario, Canada. Other chemicals and solvents were analytical or spectrographic grades.

Animals, induction of hypoglycemia. Male Wistar rats (305-345g) of a S.P.F. Wistar strain (Møllegaard Avlslaboratorium, Copenhagen) were fasted over night before the experiments with free access to water. Hypoglycemia was induced by an i.p. injection of insulin (Insulin Novo Actrapid^(R), $40\text{ IU}\cdot\text{kg}^{-1}$). The insulin was dissolved in 0.75ml of Krebs-Hensleit solution before the injection; control animals were given 0.75ml of this solution.

Anaesthesia, operative and sampling techniques. Anaesthesia was induced with 2-3% halothane about 60 min after the insulin injection; the animals were then tracheotomized, immobilized with an i.v. injection of tubocurarine chloride ($0.5\text{ mg}\cdot\text{kg}^{-1}$) and ventilated with a Starling type respirator, delivering 70 per cent N_2O and 30 per cent O_2 . One femoral artery was cannulated for continuous blood pressure recording with an electromanometer and for sampling of blood, and one femoral vein was cannulated for injections. The electrocorticogram (EEG) was continuously recorded in all animals with bipolar leads from the fronto-parietal region. After the operative procedures had been completed the animals were given Heparin i.v. (100 IU) and were maintained on 70 per cent N_2O and 30 per cent O_2 . Repeated arterial samples were anaerobically taken from the catheter in glass capillaries for determination of pH, PCO_2 and PO_2 or into liquid nitrogen for later analyses of glucose. For sampling of cerebral and cerebellar cortical tissue a skin incision was made over the skull bone for later freezing of the brain in situ with liquid nitrogen (Pontén et al. 1973).

Experimental groups. There were three groups of animals. One was a control group (n=6) maintained under anaesthesia for 93-137 min before tissue was frozen in situ. In two other groups, each containing 6 animals, the animals were given insulin and maintained with an abolished EEG activity for 30 and 60 min, respectively.

Analytical techniques. The pH, PCO₂ and PO₂ in arterial blood were measured immediately after sampling with microelectrodes operated at 37°C (Radiometer, Copenhagen, and Eschweiler and Co., Kiel). Appropriate temperature corrections were applied. Blood and tissue samples were stored at -80°C until analysis. The brains were dissected and weighed at -20°C. Cerebral tissue was extracted at this temperature as described by Folbergrová et al. (1972a). Carbohydrate substrates and labile phosphates were determined with the fluorometric techniques of Lowry and Passonneau (1972). (For analytical conditions, see Folbergrová et al. 1972a, b, 1974). Cyclic AMP was measured with a protein-binding technique and cyclic GMP was measured with the radioimmunoassay techniques described by Steiner et al. (1972a). The extraction procedure has been previously described (Agardh et al. 1978). Brain lipids were separated by thin layer chromatography. Phospholipids were eluted from the plates as described by Åkesson et al. (1970) and quantified by phosphorus determination according to the method of Belfrage et al. (1970). Total fatty acids were transesterified and free fatty acids were esterified as described by Åkesson et al. (1970). The fatty acid methylesters were separated by gas liquid chromatography. Details of extraction conditions and analytical techniques have been recently described (Agardh et al. 1981b).

Calculations and statistics. The adenylate energy charge (E.C.) was calculated according to Atkinson (1968). Statistical differences were calculated with the Mann-Whitney U-test (two-tailed). Correlations were calculated by the conventional method for linear correlation.

RESULTS

As stated above, the results pertain to 30 and 60 min of severe hypoglycemia, defined as a reduction in blood glucose

concentrations leading to cessation of spontaneous EEG activity. In none of the animals included in the series could signs of EEG activity be detected. Physiological parameters were similar to those observed previously (see Agardh *et al.* 1978, 1980, 1981a). Thus, in the hypoglycemic animals no, or only small changes could be noted in PaO_2 , PaCO_2 and pH, and blood glucose concentrations were well below $1 \mu\text{mol}\cdot\text{g}^{-1}$ (Table 1).

Table 1. Physiological parameters, arterial blood glucose and duration of anaesthesia.

	Control (n = 6)	Isoelectric EEG (min)	
		30 (n=6)	60 (n=6)
MABP (mmHg)	155(145-165)	159(137-195)	116(100-135)
PaO_2 (mmHg)	107(98-112)	100(91-106)	103(96-117)
PaCO_2 (mmHg)	37.0(35.0-39.8)	35.3(26.0-43.0)	30.9(25.0-35.1)
pH	7.39(7.34-7.43)	7.33(7.25-7.47)	7.37(7.34-7.41)
β -glucose ($\mu\text{mol}\cdot\text{g}^{-1}$) art.	5.9(5.0-6.4)	0.4(0.1-0.8)	0.7(0.5-0.9)
Anaesthesia (min)	118(93-137)	138(82-201)	154(118-193)

The values are means and ranges (in brackets).

Blood pressure was upheld in the 30 min group but fell in the 60 min group; however, no animal included had a mean arterial blood pressure below 100 mmHg.

1. Metabolites reflecting cellular energy state.

Since metabolites in cerebral cortex and cerebellum were measured in the brains of the same animals, a direct compari-

son is possible. As Table 2 shows, changes in labile organic phosphates were less pronounced in the cerebellum than in the cerebral cortex; nonetheless, deterioration of cerebellar energy state was obvious, especially after 60 min (cf. Agardh et al. 1981a).

Two further findings are worth noting: the variability in metabolic response between animals in spite of the electrical silence and the similarly reduced blood glucose concentrations, and the partial preservation of cerebellar glycogen stores. In order to allow a better comparison of cerebral cortical and cerebellar energy state, all individual values were calculated in per cent of the control values. Furthermore, since the brains were extracted on two different occasions, each hypoglycemic value was calculated in per cent of the mean of the appropriate control values (Fig.1).

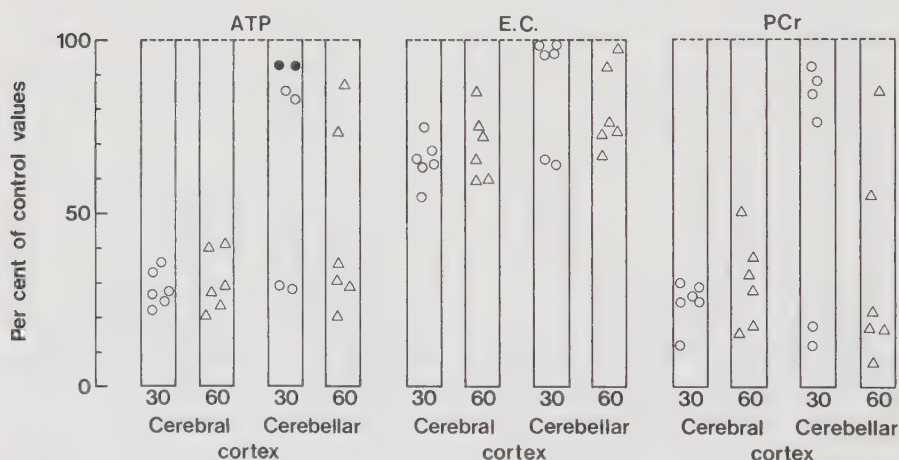


Fig.1. Individual concentrations of ATP, PCr and energy charge (E.C.) in per cent of control values in cerebral and cerebellar cortex during hypoglycemia with an isoelectric EEG for 30 and 60 min. Filled symbols indicate values within the control range.

Apart from showing the difference in metabolic response between cerebral cortex and cerebellum, the results demonstrate two

Table 2. Cerebral and cerebellar cortical concentrations of labile phosphates, carbohydrate substrates, cyclic AMP and cyclic GMP in control and during insulin-induced hypoglycemia.

	CEREBRAL CORTEX		CEREBELLAR CORTEX		
	CONTROL	HYPOGLYCEMIA (MIN)	CONTROL	HYPOGLYCEMIA (MIN)	
		30		60	30
PCr ($\mu\text{mol}\cdot\text{g}^{-1}$)	4.60 $\pm$ 0.04	1.12 $\pm$ 0.12 ^{xxx}	6.86 $\pm$ 0.13	4.20 $\pm$ 1.04 ^{xx}	2.33 $\pm$ 0.82 ^{xxx}
ATP	2.71 $\pm$ 0.02	0.76 $\pm$ 0.06 ^{xxx}	2.64 $\pm$ 0.06	1.81 $\pm$ 0.34 ^{xx}	1.22 $\pm$ 0.30 ^{xxx}
ADP	0.334 $\pm$ 0.009	0.771 $\pm$ 0.104 ^{xxx}	0.260 $\pm$ 0.010	0.418 $\pm$ 0.063 ^{xxx}	0.429 $\pm$ 0.031 ^{xxx}
AMP	0.055 $\pm$ 0.010	0.370 $\pm$ 0.048 ^{xxx}	0.051 $\pm$ 0.004	0.179 $\pm$ 0.068 ^{xx}	0.166 $\pm$ 0.025 ^{xx}
Energy Charge	0.928 $\pm$ 0.004	0.604 $\pm$ 0.026 ^{xxx}	0.939 $\pm$ 0.003	0.811 $\pm$ 0.066 ^{xxx}	0.759 $\pm$ 0.045 ^{xxx}
Glucose	2.14 $\pm$ 0.11	0.15 $\pm$ 0.02 ^{xxx}	3.09 $\pm$ 0.13	0.16 $\pm$ 0.04 ^{xxx}	0.25 $\pm$ 0.05 ^{xxx}
Pyruvate	0.084 $\pm$ 0.006	0.027 $\pm$ 0.004 ^{xxx}	0.049 $\pm$ 0.004	0.024 $\pm$ 0.003 ^{xxx}	0.026 $\pm$ 0.005 ^{xx}
Lactate	1.22 $\pm$ 0.19	0.59 $\pm$ 0.21	0.79 $\pm$ 0.05	0.21 $\pm$ 0.06 ^{xxx}	0.24 $\pm$ 0.04 ^{xxx}
Cyclic AMP ($\mu\text{mol}\cdot\text{kg}^{-1}$)	1.84 $\pm$ 0.10	2.84 $\pm$ 0.38 ^{xx}	1.46 $\pm$ 0.20	5.39 $\pm$ 1.31 ^{xxx}	7.23 $\pm$ 1.68 ^{xxx}
Cyclic GMP ($\mu\text{mol}\cdot\text{kg}^{-1}$)	0.07 $\pm$ 0.01	0.16 $\pm$ 0.01 ^{xxx}	0.90 $\pm$ 0.22	4.50 $\pm$ 2.10	6.13 $\pm$ 1.27 ^{xxx}

The values are means $\pm$ S.E.M. ^x = $p < 0.05$; ^{xx} = $p < 0.02$; ^{xxx} = $p < 0.002$ (MANN-WHITNEY U-TEST).

things. First, in the 30 min group two cerebellar ATP values fell within the control range but this was evidently due to the presence, in the control group, of one single aberrant value ($2.37 \mu\text{mol}\cdot\text{g}^{-1}$). Thus, all calculated energy charge values were below the control range (see also PCr concentrations). The data also show that 4 of 6 animals had an energy state that was only moderately perturbed, while gross energy failure was observed in the remaining two. Second, after 60 min of hypoglycemia, all these variables illustrated were well outside the control range but one animal had a surprisingly small metabolic perturbation. This variability is in confirmation of our previous results (Agardh *et al.* 1981a).

The results of Fig.2 demonstrate that in more than half of the animals the cerebellar glycogen content remained at values exceeding 10 per cent of control. The data also reveal a striking correlation to the adenylate energy charge. We conclude from these results that gross deterioration of cerebellar energy state does not occur until glycogen concentrations are reduced below about $0.5 \mu\text{mol}\cdot\text{g}^{-1}$. Under similar conditions (*i.e.* 30 and 60 min of hypoglycemic coma) glycogen concentrations in the cerebral cortex are reduced to $0.1 \mu\text{mol}\cdot\text{g}^{-1}$, or lower. The results suggest, therefore, that cerebellum receives a better substrate supply than the cerebral cortex (see Discussion).

2. Cyclic nucleotides.

Results on cyclic nucleotides are shown in Table 2. For the cerebral cortex, the results on cGMP corroborate those reported previously (Agardh *et al.* 1978) and extend to the observations to 60 min of hypoglycemia. However, the results on cAMP, which demonstrate a significant elevation both at 30 and 60 min, are seemingly discordant with our previous data which suggested that cAMP returns to control concentration after 30 min of hypoglycemia (Agardh *et al.* 1978). The results of Fig. 3 provide an explanation. Thus, in the energy charge range 0.5-0.8 cAMP concentrations were directly correlated to energy charge ($p < 0.001$). In other words, a secondary decrease in cAMP concentrations to control values only occurs when the energy charge is reduced to below about 0.6, *i.e.* to values ob-

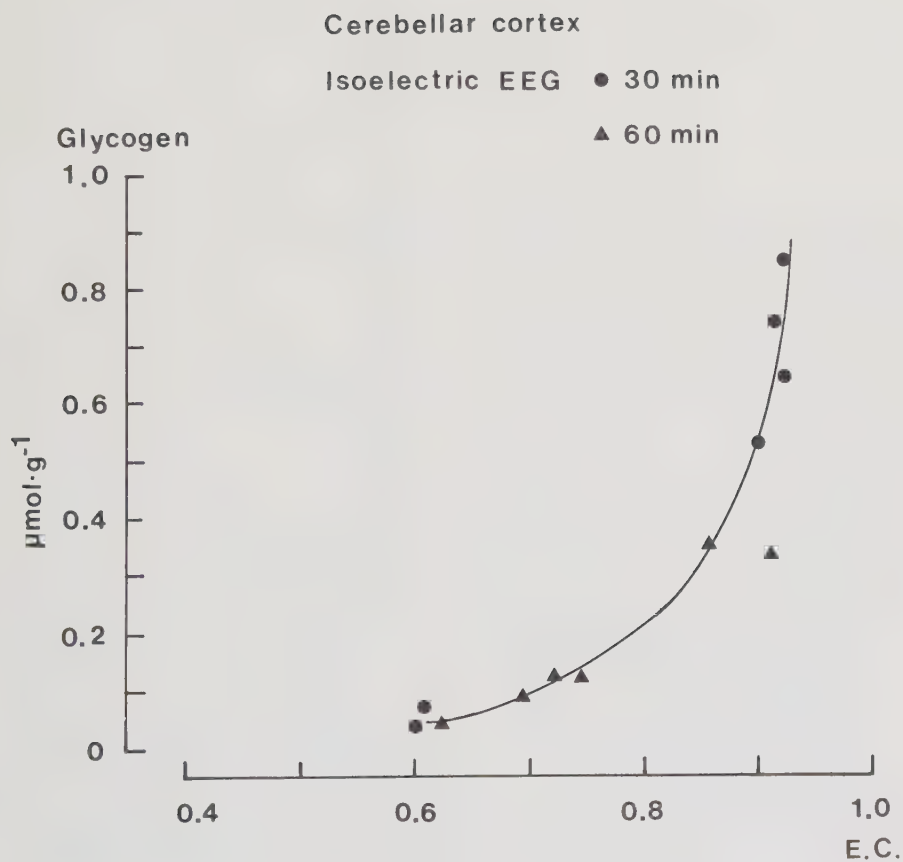


Fig.2. Relationship between cerebellar cortical concentration of glycogen and the energy charge (E.C.) of the tissue. The line is drawn for visual best fit.

CEREBRAL CORTEX

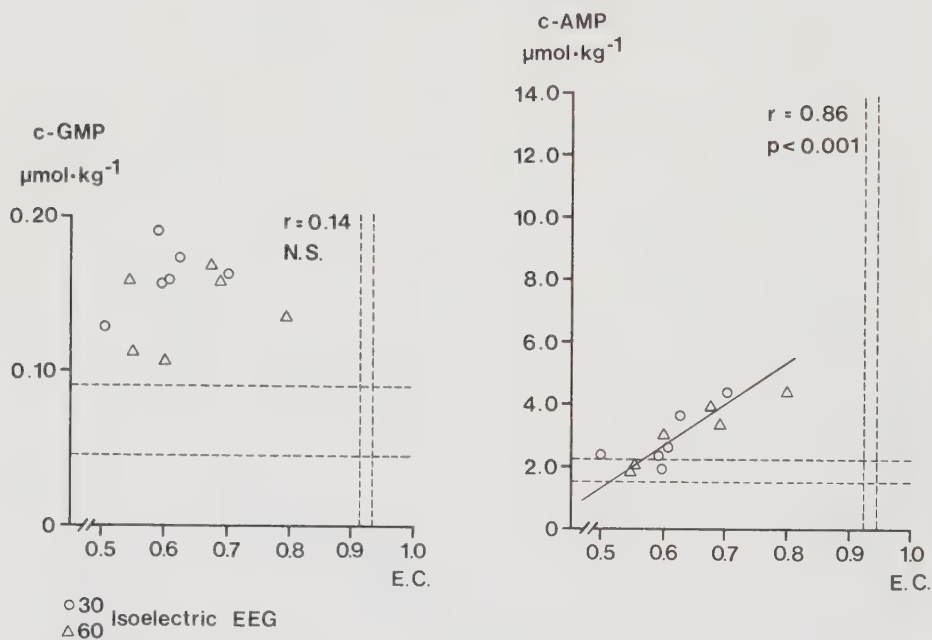


Fig.3. Relationship between cerebral cortical concentrations of cyclic GMP (left), cyclic AMP (right), and the energy charge (E.C.) of the tissue. Interrupted lines represent range of control.

served in the previous study (Agardh et al.1978). A corresponding correlation for cGMP reveals an appreciable scatter. Thus, although values during hypoglycemia were clearly increased, they showed no significant correlation to energy charge. Obviously, the two cyclic nucleotides behave differently when cerebral energy state is progressively decreased.

A corresponding analysis of cerebellar changes reveals interesting differences from those observed in cerebral cortex (Fig.4).

CEREBELLAR CORTEX

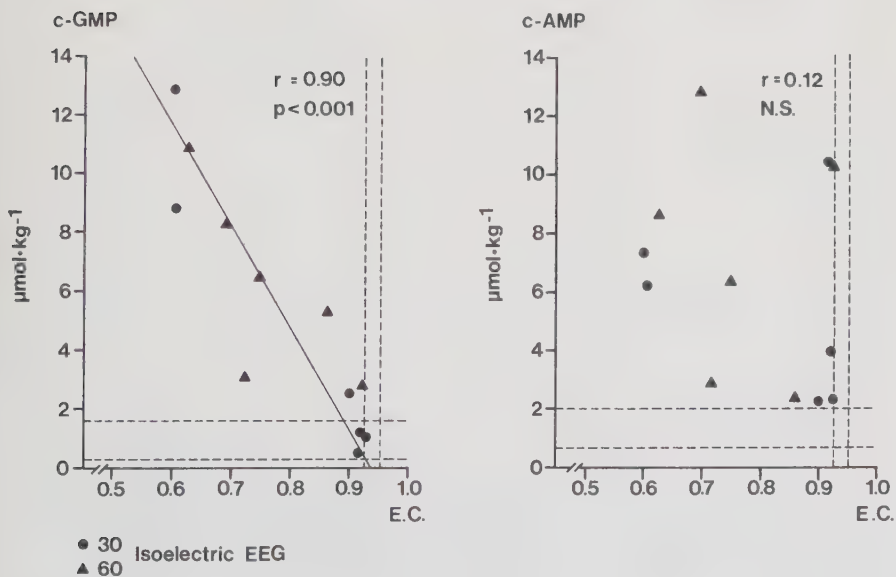


Fig.4. Relationship between cerebellar cortical concentrations of cyclic GMP (left), cyclic AMP (right) and the energy charge (E.C.) of the tissue. Interrupted lines represent range of control.

First, although the cerebellar cAMP concentrations were poorly correlated to the energy charge, they were considerably higher than those recorded in the cerebral cortex, and the values

showed no clear tendency to decrease at low energy charge values. Second, the cyclic GMP values, which showed a tight relationship to the energy charge, rose to higher values than in the cerebral cortex.

3. Phospholipids, phospholipid-bound fatty acids, and free fatty acids.

Previous results from the laboratory (Agardh *et al.* 1981b) have shown that, in the cerebral cortex, hypoglycemia of 30 min duration is associated with a moderate (8-10 per cent) decrease in phospholipid phosphorus and fatty acid content. Results on cerebellum are shown in Table 3. As observed, hypo-

Table 3. Total fatty acids, total phospholipid phosphorus concentrations, and free fatty acids in rat cerebellar cortex in control, and during hypoglycemia with an isoelectric EEG for 30 and 60 min.

	Control (n=6)	Isoelectric EEG (min)	
		30 (n=6)	60 (n=6)
Total fatty acids	111.9 $\pm$ 4.5	110.6 $\pm$ 4.9	103.2 $\pm$ 2.6
Total phospholipid phosphorus	70.8 $\pm$ 3.1	65.2 $\pm$ 1.3	63.1 $\pm$ 2.3
Total free fatty acids	0.206 $\pm$ 0.17	0.402 $\pm$ 0.93	0.519 $\pm$ 0.081*

The values are given in $\mu\text{mol}\cdot\text{g}^{-1}$ cortex wet weight and represent means $\pm$ SEM.

* = $p < 0.05$ (Mann-Whitney U-test)

glycemia of 30 and 60 min duration did not lead to significant changes in phospholipid phosphorus or total fatty acid content, and the increase in FFA was significant only after 60 min. However, the lack of changes in the first two of these variables seemed to be due to the variability in response. Thus, if data from animals with an energy charge higher than 0.85 were pooled and compared with those with an energy charge exceeding that value (n=6 in each group) hypoglycemia was found to be associated with a significant reduction in phospholipid phosphorus (the values were 61.3 ± 1.4 and $67.1 \pm 1.4 \mu\text{mol}\cdot\text{g}^{-1}$, respect-

ively; $p < 0.02$). One control value was grossly aberrant ($84.3 \mu\text{mol} \cdot \text{g}^{-1}$). If that was excluded the mean control value ($68.1 \pm 1.9 \mu\text{mol} \cdot \text{g}^{-1}$) was very close to that derived for hypoglycemic animals with an energy charge exceeding 0.85 ($67.1 \pm 1.4 \mu\text{mol} \cdot \text{g}^{-1}$). A corresponding analysis of results on fatty acid contents showed that values obtained in animals with an energy charge below 0.85 were only 3 per cent lower than in the remainder; besides, this difference was not significant. Thus, a decrease in cerebellar phospholipid content is only suggestive.

Since the main emphasis on lipid analysis was laid on FFA these were analysed in both the cerebral cortex and the cerebellum. Fig.5 shows that in spite of the fact the control ce-

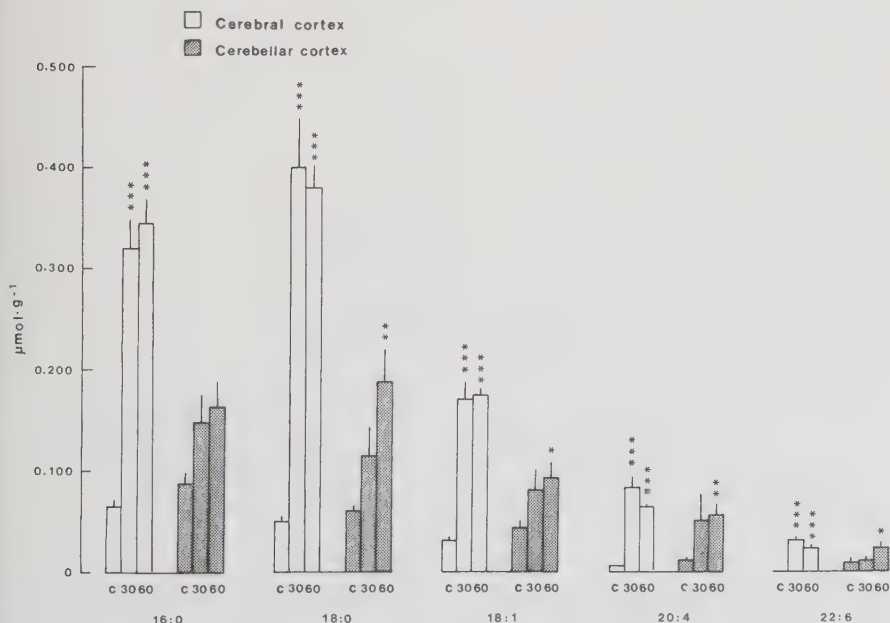


Fig.5. Cerebral and cerebellar cortical concentrations of individual free fatty acids in controls (C) and after hypoglycemia with an isoelectric EEG for 30 and 60 min. (16:0 palmitic acid; 18:0, stearic acid; 18:1, oleic acid; 20:4, arachidonic acid; 22:6, docosahexenoic acid). Values are means $\pm$ S.E.M. In $\mu\text{mol} \cdot \text{g}^{-1}$ wet tissue weight. Significant increase in experimental group compared with control group: * $p < 0.05$, ** $p < 0.02$, *** $p < 0.002$, (Mann-Whitney U-test)

rebellar concentrations of individual (or total, see Table 3) FFAs were higher than in the cerebral cortex, the cerebellum showed lower concentrations of individual FFAs during hypoglycemia. As in the cortex, though, the largest relative change occurred in the concentration of arachidonic acid (20:4).

Although the results of Fig.5 demonstrate that, in hypoglycemia, smaller amounts of FFA accumulate in the cerebellum than in the cerebral cortex, the data do not indicate whether or not this difference is related to differences in energy state. We related, therefore, individual energy charge values to total FFA content. Since the control values were different, all values were calculated in per cent of control (Fig.6).

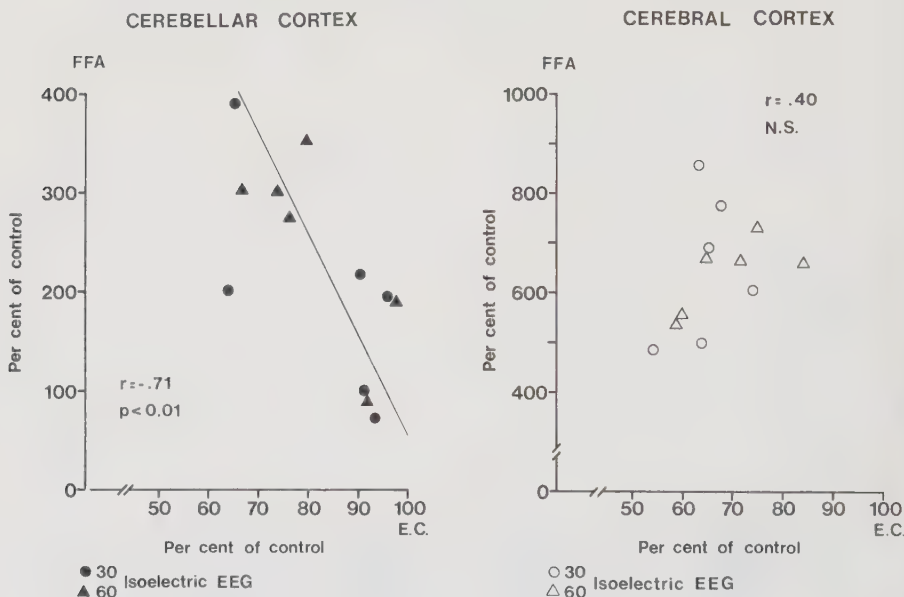


Fig.6. Relationship between cerebellar cortical (left) and cerebral cortical (right) concentrations of total fatty acids (FFA) and the energy charge of the tissue. The values are given in per cent of control.

In the cerebral cortex, no relationship was found between energy charge and FFA content; however, the range of energy charge values was small (0.55 - 0.85). In the cerebellum, a highly significant correlation was found between the energy charge

and the FFA content.

The data reveal that for a given reduction in energy state, accumulation of FFA was apparently more pronounced in the cerebral cortex than in the cerebellum (observe difference in scales).

DISCUSSION

The present study has given four main results. First, in confirmation of a previous study (Agardh et al. 1981a) the data show that, in hypoglycemia, the deterioration of cellular metabolism has a slower onset and is less pronounced in the cerebellum than in the cerebral cortex. However, in all animals some deterioration of cerebellar energy was observed already after 30 min of hypoglycemia and, after 60 min, the majority of animals showed signs of extensive energy failure. Second, in animals showing gross deterioration of cellular energy state mobilization of endogenous substrates seemed to involve loss of phospholipids, similar to that previously noted in the cerebral cortex. Third, in the cerebellum (as compared with the cerebral cortex) hypoglycemia was accompanied by a more marked accumulation of both cGMP and cAMP, the latter showing no tendency to decrease in concentration with progressive reduction in energy charge. Fourth, although FFA (and arachidonic acid) accumulated in the cerebellum during hypoglycemia, the accumulation was less pronounced than in the cerebral cortex, even when the comparison was made at similar degrees of energy failure. We will discuss the main implications of these results.

1. Substrate supply to the cerebellum.

The present results, and those published previously (Agardh et al. 1981a) strongly suggest that the cerebellum receives a better supply of exogenous substrate than the cerebral cortex. Thus, although the cerebellum contains somewhat higher stores of glycogen and glucose than the cerebral cortex the difference (about $2 \mu\text{mol}\cdot\text{g}^{-1}$ in terms of glucose equivalents) cannot support oxidation for more than a few minutes (rate of glucose utilization about $0.55 \mu\text{mol}\cdot\text{g}^{-1}\cdot\text{min}^{-1}$, see Abdul-Rahman and Siesjö 1980). Furthermore, endogenous stores of other carbohydrate metabolites and of amino acids do not

exceed those of the cerebral cortex (Agardh et al. 1981a). We are then left with the possibility that glucose transport to the cerebellum exceeds that to the cerebral cortex. Evidence for that has been obtained in experiments in which glucose delivery was estimated by the ^{14}C -deoxyglucose technique of Sokoloff et al. (1977) and Savaki et al. (1980). In these experiments, provisional lumped constants were used, the value employed for hypoglycemic coma being 0.87 (Abdul-Rahman and Siesjö 1980). Subsequent results have shown that when tissue glucose concentrations are reduced to below about $0.5 \mu\text{mol}\cdot\text{g}^{-1}$ the lumped constant increases towards a limiting value of 1.4 (Crane et al. 1980). In the present 30 and 60 min groups, all tissue glucose concentrations (cerebral cortex and cerebellum) were below 0.40, and the majority were below $0.20 \mu\text{mol}\cdot\text{g}^{-1}$. Thus, the previous local glucose utilization rates (Abdul-Rahman and Siesjö 1980) may represent overestimations of the true ones. However, since there is no reason to suspect that the lumped constant is different for cerebellum and cerebral cortex (see tissue glucose values in Table 2) we are left with the finding that, at the same plasma glucose concentration, the cerebellum receives a better glucose supply than the cerebral cortex. Tentatively, this reflects a higher density of vascular transport sites for glucose in the cerebellum (see Abdul-Rahman and Siesjö 1980). Whatever the explanation, a more efficient glucose transport must contribute to the fact that metabolic deterioration has a slower onset, and is less extensive, in the cerebellum than in the cerebral cortex. Undoubtedly, this property must contribute to the resistance of cerebellar cells to the insult of severe hypoglycemia.

2. Cyclic nucleotide metabolism.

It is of interest that although the cAMP concentration rises in both cerebral cortex and cerebellum during hypoglycemia, the concentration is subsequently reduced in the cerebral cortex but not in the cerebellum (Agardh et al. 1978, and present results). In the cortex, the secondary reduction is obviously related to progressive energy failure (Fig.3). However, in view of the fact that cGMP concentrations are not reduced (see below) and that decreases in the reduction of ATP

and GTP levels during hypoglycemia are not grossly dissimilar (Chapman et al. 1981), it appears less likely that cAMP values fall due to depletion of substrate (cf. also difference in cerebral cortical and cerebellar levels of cAMP at comparable degrees of ATP depletion). Current views favour catecholamines notably noradrenaline, and adenosine as the main activators of adenylate cyclase (see Bloom 1975, Iversen 1977, Ferrendelli et al. 1980). Since catecholamines are released during hypoglycemia (Agardh et al. 1979) and adenosine accumulates (Chapman et al. 1981) the secondary reduction in cAMP concentration represents an enigma. However, since noradrenaline has been shown to mediate inhibition of pyramidal cells in the cerebral cortex (Bloom 1975, Stone 1977) and Purkinje cells in the cerebellum (Hoffer et al. 1971) it is conceivable that the cAMP data reflect differences in inhibitory tone. Although speculative, this possibility is of interest in view of the fact that hypoglycemia causes damage of cerebral cortical pyramidal cells but not of cerebellar Purkinje cells.

There is now considerable evidence that Ca^{2+} is the main activator of guanylate cyclase (see Ferrendelli et al. 1976, Goldberg and Haddox 1977, Briggs and DeRubertis 1980). However, Ca^{2+} does not cause accumulation of cGMP in the absence of oxygen (see Briggs and DeRubertis 1980), as is also evidenced by the fact that cGMP concentrations are reduced during ischemia and increase markedly in the recirculation period (Steiner et al. 1972b, Kobayashi et al. 1977). The oxygen dependence of the Ca^{2+} activation of guanylate cyclase has been ascribed to the fact that some of the most important activators of this enzyme are hydroperoxide breakdown products of polyenoic fatty acids (Asano and Hidaka 1977, Murad et al. 1979). Thus, while an increase in free Ca^{2+} activity in the cell must exist in all conditions leading to energy failure, activation of guanylate cyclase does not ensue until such acids, released by the activation of phospholipase A_2 , has been oxidatively degraded. If this is so, we would expect a relationship between free fatty acid concentration and cGMP. However, the comparison breaks through when we compare cerebral cortex and the cerebellum, the latter tissue showing higher cGMP and low-

er FFA concentrations. Possibly, the guanylate cyclase systems of these two structures are dissimilar.

3. Changes in free fatty acids.

As discussed in more detail elsewhere (Siesjö 1981) accumulation of FFA, especially arachidonic acid, is supposed to trigger a series of adverse reactions. These include inhibition of adenine nucleotide translocation across the inner mitochondrial membrane (Wojtczak 1976, Shrago 1979), inhibition of mitochondrial respiration (Pressman and Lardy 1956, Björntorp et al. 1964, Lochner et al. 1978) and uncoupling of oxidative phosphorylation (Pressman and Lardy 1956, Lehninger and Remmert 1959, Björntorp et al. 1964, Vázquez-Colón et al. 1966, Wojtczak 1976, Chefurka 1966). Other effects of increased FFA concentrations involve cellular swelling in brain slices (Chan and Fishman 1978), and reactions secondary to increased formation of fatty acid cyclo-oxygenase products (Gaudet and Levine 1979, Hallenbeck and Furlow 1979) and lip-oxygenase products (Murphy et al. 1979, Borgeat and Samuelsson 1979).

In view of all these potentially adverse effects of (poly-enoic) fatty acids it is of interest that cerebellum (a 'resistant' structure) accumulated less FFA than the cortex (a 'vulnerable' structure). Undoubtedly, part of this difference must have been due to the fact that the cerebral cortex suffered greater metabolic damage than the cerebellum. However, even if the two tissues were compared at similar degrees of energy failure, less FFA (and arachidonic acid) accumulated in the cerebellum. We tentatively conclude, therefore, that there may be differences in Ca^{2+} balance or in phospholipase activities between the two tissues which may well contribute to the greater resistance of cerebellar cells to the hypoglycemic insult.

ACKNOWLEDGEMENTS

The authors are grateful to Kerstin Beirup, Karin Hansson, Mette Lindell and Lena Sjöberg for their excellent technical assistance, and to Gillian Sjö Dahl for typing the manuscript. This study was supported by grants from Segerfalk Foundation, The Swedish Medical Research Council (project No.14X-263) and

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